A study of the mechanisms of antagonism by the biocontrol fungi *Trichoderma* against wood decay basidiomycetes

Usha Srinivasan BSc. (Hons).

This thesis is presented to Dundee Institute of Technology in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy.

Scottish Institute of Wood Technology, Department of Molecular and Life Sciences, Dundee Institute of Technology.

September 1993

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This manuscript is dedicated to my thatha, appa, amma, uma kutty and my karadi

Acknowledgements

My sincere gratitude and thanks goes firstly to Dundee Institute of Technology for the financial support of this project.

Sincere thanks to my supervisor Dr. A. Bruce for the timeless hours that he has spent with me (excluding the talks on 'Hibs') during the course of the three years of my Ph.D., helping me in every aspect of my work, without which I would not be at this stage of completion. I would also like to thank Dr. Highley (Project leader of the Biocontrol and Biodegradation, Forest Products Laboratory, Madison, Wisconsin, U.S.A.), for his valuable guidance and financial support during my six month research programme in his laboratory. I must also thank the other researchers in the Forest Products Laboratory, who in one way or another made my trip memorable : Rick for giving me the attentions of a "princess", Carol for enduring me and Rick, Suki for help with the basidiospores and Larry for help with the NMR. I owe a very special thanks to Harry Staines for being such an immense help with the stats, and being so patient with biologists such as me.

Thanks to Dr. Bremner for help with my NMR interpretations. Thanks are also due to staff of the media centre for help with photographic assistance. Technicians for enduring my constant pleas for help, especially Willie. Anne, who though not with us now, had given me immense moral support and encouragement without which I could not have endured the first year of my Ph.D.

Particular credit must be extended to my fellow research students for their endurance and patience throughout the write up of this document and for letting me be in my own world. Special thanks to Nia for letting me pick her brain during the last few hectic months.

Finally, I will always be indebted to my parents for their continuing love, support and encouragement, whithout which the completion of this manuscript would not have been possible. And my 'karadi' for constant comfort during difficult times.

Abstract

Environmental concern regarding the use of hazardous wood preservatives is of increasing concern, and biological control may provide an attractive alternative to the use of toxic chemicals for wood protection. ***Trichoderma*** isolates are among the most widely researched biological control agents for the protection of agricultural crops from a variety of plant diseases and have more recently been investigated for bioprotection of wood products.

Screening of fungal antagonists is the first most important step in selection of potential biocontrol agents. Preliminary interaction studies were carried out between ***Trichoderma*** spp. and selected basidiomycetes on both nutrient rich media and a low nutrient media devised to give a closer representation of the nutritional consistency of wood. Results indicated that the outcome of interactions was dependent on the media type.

Individual antagonistic mechanisms of ***Trichoderma*** (soluble metabolite, volatile antibiotics, laminarinase and chitinase lytic enzymes) against ***Neolentinus lepideus*** and ***Trametes versicolor*** fungi indicated that these were also dependent on media type. Study of production of iron chelating siderophores by ***Trichoderma*** species indicated that they may also play a significant role in antagonism against basidiomycetes by iron competition.

One of the major aims of the work was to identify the relative importance of individual antagonistic mechanisms of ***Trichoderma*** that would be of importance during interaction with wood decay basidiomycetes in the natural substrate, i.e., wood. Statistical comparisons were carried out between the % weight loss of wood blocks after exposure to selected basidiomycetes, with individual antagonistic responses observed against these same wood decay fungi using agar test systems. Results indicate that the relative importance of individual antagonistic responses exhibited by ***Trichoderma*** spp. in determining the degree of wood protection is dependent on the following factors: 1) target pathogen, 2) ***Trichoderma*** spp. and 3) the media in which the responses were

detected. This project has important commercial implications since through a better understanding of the strategies of the antagonists, strains can be developed to exaggerate their more important antagonistic mechanisms or to better target, specific decay fungi. Also knowledge gained on the potential of certain metabolites and volatiles can be adapted in commercial production of safer wood protectants which may replace toxic chemical preservatives currently in use.

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Abbreviations

MEA	Malt extract agar
MEB	Malt extract broth
LNM	Low nutrient media
LNM+G+CW	Low nutrient media with same amount of glucose as LNM but with basidiomycete cell wall material
LNM-G+CW	Low nutrient media without glucose but with basidiomycete cell wall material
EDTA	Ethylenediamino-tetra-acetic acid
P	Phenolate siderophores
H	Hydroxamate siderophores
CAS	Chrome azurol S agar
TLC	Thin Layer Chromatography
DMAB	p-Dimethylaminobenzaldehyde

Chapter 1

Introduction

Chapter 1

1.0 Introduction

A considerable amount of money is lost annually worldwide because of the biodeterioration of wood products by micro-organisms. A large proportion of this money could be saved by the use of suitable wood species and correct selection of available control methods. Certain species of timber are naturally resistant to attack because they contain toxic heartwood extractives and perform well in service without the addition of chemical preservatives (Panshin and de Zeeuw, 1980). Unfortunately, natural durability is variable even within and between trees of the same species, and naturally durable timbers are becoming progressively more scarce and correspondingly more expensive as the demand for them is increasing. Consequently, it is generally becoming more economical to use properly protected nondurable timber rather than naturally durable material.

The two major control methods for protection of wood in service are 1) prevention of the entry of moisture into wood by the use of proper construction techniques and water-impermeable barriers, and 2) treatment of wood with chemicals toxic to wood-destroying fungi. The former method is widely used in low-hazard environments; for example, internal building timbers in temperate zones. The latter method is used in medium- and high-hazard environments; for example, ground-contact timbers and marine timbers (Tsoumis, 1991).

While preservatives have been used for some time now awarness of their environmental hazards has greatly increased in recent years. Alternatives are now being sought that will be environmentally safer as well as effective. Biological control is one such alternative. The idea of using one organism to control attack by another is not new in the field of biological sciences. In fact, it has become a practical reality for the control of some insect pests and some microbial pathogens which attack a range of plant species.

However, to date the development of fungal control methods for prevention of decay in wood has not been fully exploited.

1.1 Biological control: Understanding the phenomenon

Biological control is part of a broader overall phenomenon of natural control. Natural control is common to all biological systems and occurs as part of the ecology of individual environments. Natural control may be defined as the regulation of populations over a period of time by any single or combination of natural factors. Such factors have sometimes been classed into two categories biotic (living) and abiotic (non-living). For example, weather can have an abiotic regulating effect in insect population densities by interacting with other physical factors. For instance, the higher the temperature and lower the humidity, the fewer are the sites on a plant that a phytophagous (plant eating) insect may be able to survive. Although any single factor may be the key regulatory component responsible for control in a given situation rarely if ever do they act entirely independently. And abiotic factors will interact with biotic aspects of a habitat, where, though temperature can affect the insect population, its reduction in turn might affect populations of others that feed on them (DeBach, 1974). Biological control was defined by DeBach (1974) as " the regulation by natural enemies of another organism's population density to a lower level than would otherwise occur". This definition of biocontrol includes both natural control where control has occurred as a result of the accidental immigration and establishment of an exotic natural enemy; or alternative biological control which is achieved through human activities. Since a vast amount of biological control is natural, it is therefore reasonable to consider cases involving manipulation by man to achieve control as being " applied or classical biological control ".

Primitive man must have observed, in very early times, the delicacy of balance in nature by noting that excessive hunting of an animal lowered its numbers and thus decreased his own food supply. Strategies therefore evolved which attempted to maintain a favourable balance. Man has been learning through time that such a fine balance in nature is difficult to maintain. Domestic practices such as controlling pests like mice and

rats with cats is a perfectly simple example to demonstrate man's earlier efforts to develop biocontrol. With the progress of civilisation, humans have without knowledge or insight into the mechanisms of biological control, practiced limited biocontrol through the practices of crop rotation and mixed or inter-cropping methods in the Middle East and later in China in the third and fourth millennia BC. The Europeans in Saxon and medieval times used simple rotation and fallow periods to try to reduce disease and increase soil fertility (Campbell, 1989 a). Only more recently the remarkable interactions that are responsible for such control have been analysed and explored. Such biological control strategies, subjected to adequate research, are applicable to systems containing almost all life forms. Inorder to maintain a balanced control system, great care has to be taken particularly when the control agent is not already endemic in that ecosystem. The introduction of the myxoma virus to control rabbit population in Australia and the introduction of a north Argentinian native moth ***Cactoblastis cactorum*** to control prickly pear, a cactus which became a serious weed in Australia have both been well documented failures due to excessive population explosions of the introduced control agents (Deacon, 1983). There is a fine dividing line between successful biocontrol of populations and the forced extinction of the target. In many instances human manipulation of populations of organisms in the form of applied biological control has caused irreparable damage however increasing awareness of the hazards of applying insufficiently analysed biological control strategies has prompted extensive research in this area.

Data from a global appraisal of the success rate of classic biological control against insect and arachnid pests (Hall and Ehler, 1979), shows that only one-third of the parasites and predators introduced become established more or less permanently after introduction. Since most of the earlier work has involved biocontrol of the above mentioned pests, some valuable knowledge gained in terms of introduction and success rates of biocontrol agents has been gathered from this study and is discussed below. Indeed if success of biological control is defined purely in terms of profitability, only 16 percent of these classic biological control attempts qualify as successes (Hall ***et al.***,

1980), while Turnbull and Chant (1961) concluded that well over half of the Canadian biological control projects were failures.

A number of theories have been proposed on the basis of experimental experience to account for such failure of insect biological control agents (Krebs, 1985). Most successfully implemented biological control programs have given early indications that they were likely to succeed. Clausen (1951) suggests that three years should be a large enough period to establish success and that if control is not achieved in the vicinity of the colonisation point within this time, the complete control by a biocontrol system will be most unlikely. This suggests that projects where the control agent does not become established should be discontinued after three years and that prolonged efforts at establishment beyond this time are not economically practical. Most biological control agents of insect pests which successfully established within the three year rule, show little subsequent evolutionary change beyond this span of time. If an antagonist is not already adapted to control the pathogen it will not evolve quickly into a successful control agent.

A biocontrol agent selected on the basis of antagonistic and ecological characteristics as determined by laboratory experiments may still fail during field testing however, if commercial exploitation of such systems is to occur thorough preliminary experimentation has to be undertaken. Pest management in the form of integrated control with chemicals and biocontrol agents has shown promising results and may provide a more immediate solution to modern problems of pest control. Toxic chemical treatments are gradually being replaced with biocontrol or integrated control systems that cause minimal damage to the environment. To replace all current hazardous treatments with biological control is however a noble but worthy challenge.

1.2 Historical development of biological control

The science of biological control began 100 years ago (DeBach, 1974) and is essentially a direct result of gradual accumulation of biological and ecological knowledge as man's civilization has progressed. The history of biological control is therefore the history of

knowledge contributed by early naturalists, biologists and experimental scientists. Biological control initially developed as an offshoot of the science of entomology for pest control. The first use of predatory insects by man for the purpose of biological control was by the ancient Chinese. They fostered the ant ***Phylla smaragdina*** in their citrus trees to control caterpillars and large boring beetles. This predatory ant builds great paper nests in trees containing thousands of individuals. These could be either purchased or recovered from wild trees and the movement of ants between cultivated trees was also encouraged by placing bamboo runways from one tree to another. A similar development was recorded among the Yemenese date growers of Arabia by P. Forskal in 1775 (cited in DeBach, 1974).

The first known successful introduction of a natural enemy from one country to another occured around the same time and indicates that the idea of using predators may have been a common practice at that time. The red locust, ***Nomadacris septmfasciata***, was the most serious agricultural pest in Mauritius. In order to possibly solve the problem the mynah bird was introduced from India by the Count de Maudave in 1762 and by 1770 the successful control of the locust was achieved. Meanwhile, in Europe during the entire seventeenth century biocontrol had developed from the first vague observations of insect parasitism to a full understanding of the process. U. Aldrovardi in 1602, was the first person to observe insect parasitism where he recorded the existence of parasitic larvae of ***Aparteles glomeratus*** in the common cabbage butterfly larvae (cited in DeBach, 1974). This led to an increased knowledge of parasites as biocontrol agents.

In the nineteenth century, biology, including biological control, came of age. By this time it was recognised that true fungi actually grew in the bodies of some insects, some clearly as saprophytes but others, as parasites. Meanwhile beginning about the mid-1800s the idea of using micro-organisms to control insects began to be considered. This concept grew out of the increasing knowledge that insect diseases were infectious and contagious and could be transmitted from diseased to healthy individuals.

Agostino Bassi is considered the father of insect pathology and has contributed a considerable amount of knowledge with regard to insect diseases and in 1836 published a suggestion to utilize putrified liquids to spray the leaves of plants to kill pest larvae. In the meantime, workers in Russia published reports on production of parasitic fungal spores on sterilised beer mash as a means of insect control. In 1884, in Smela, Russia experiments were led by Isaak Krassilstschik to develop a small plant for the production of ***Metarrhizium*** spores which led to increased interest in the use of fungi to control insects. Similarly development of control systems directed against weeds were studied around the 1850's. The American entomologist Asa Fitch was the first to suggest the biological control of weeds about 1855 when he observed that toad-flax, a European weed very destructive in New York pastures, had no American insects feeding on it and speculated that the importation of insects feeding on it in Europe might solve the problem (cited in DeBach, 1974).

Control systems employing insect parasites and predators developed considerably faster than those using microbial pathogens during the 1800s, due to the technical difficulties of studying minute pathogenic micro-organisms. Dr. Erasmus Darwin, by stressing the controlling effect of certain parasites suggested their use in agriculture and gardening. Foreign exploration for new exotic natural enemies of insects however remained as the classical approach to their biological control particularly during the early 1900's (DeBach, 1974).

One such project was the control of cottony-cushion scale a small coccid insect that sucks sap from leaves and twigs of citrus trees in California, in 1888-9. In 1888 Albert Koebele of the Division of Entomology was sent to Australia by the U.S. government to represent the State Department at an international exposition in Melbourne. Koebele sent two insects back to California, a small dipteran parasite, ***Cryptochaetum iceryae***, and a predaceous ladybird called the vedalia beetle (***Rodolia cardinalis***) as two potential control agents for the scale problem. In late 1888 the first ladybird beetles were received in California, and by January 1889 a total of 129 individuals had been released near Los Angeles. By October 1889, scarcely one year since ***Rodolia*** was found in Australia by

Koebele, the cottony-cushion scale was virtually eliminated from large areas of citrus orchards in southern California (Krebs, 1985).

In more recent years biological control has also been examined in agricultural systems for control of plant pathogens. The major reason for the interest in this field is due to the increasing need for environmentally safer pest and disease control methods. These systems must be compatible with the ever-increasing need for food, especially for improved crop production in the developing countries to achieve higher yields without causing more damage to their fragile environment. Similarly environmental awareness in the use of toxic wood preservatives has forced the need for research into safer alternatives such as biocontrol.

1.3 Microbial control and biological control : definitions and scope

DeBach (1964) defined biocontrol as " the action of predators, parasites, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence". This referred particularly to insect pests and weeds but is not broad enough to encompass control of plant pathogens, which is often achieved through more passive mechanisms. Plant pathologists have adopted an extremely broad view of biological control and include, for example, all cases in which a plant is bred for disease resistance, i.e. by genetic manipulation. Baker and Cook (1974), defined biological control as " the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists". This definition concentrates more on the specific mechanisms of control rather than defining an overall strategy for control. The definition proposed by Garrett (1970) however is even more general and covers both pests and pathogens of plants, " biological control is the practice in which, or process whereby, the undesirable effects of an organism are reduced through the agency of another organism that is not the host plant, the pest or pathogen, or man". In

other words biological control is mediated by a "third party"; in the case of microbial control this is a micro-organism.

The simplest form of microbial biological control would involve applying the control agent to reduce the effects of a target pest. There are many such cases in current practice as reviewed by Deacon (1983) including the use of ***Bacillus popilliae*** on grass turf in the USA to control larvae of the Japanese beetle and application of ***Peniophora gigantea*** to pine stumps in Britain to prevent them from being colonised by the aggressively pathogenic fungus ***Heterobasidion annosum***.

Not all examples of successful biological control involve the direct introduction of the control agents. If cereals are grown continuously (i.e. year after year) on the same site they may at first be heavily diseased by the take-all fungus ***Gaeumannomyces graminis var. tritici*** but the level of disease declines in subsequent years and cereals can be grown profitably on these "take-all decline" sites. It is known that micro-organisms, particularly some fluorescent pseudomonas bacteria, may be responsible for the take-all decline which is being exploited. In the greenhouse cropping industry it is common practice to partly sterilize soils by steam-air mixtures or chemical fumigants but soils are intentionally not completely sterilised. Partial sterilisation is sufficient to kill most weed seeds and plant pathogens but it leaves some of the resident saprophytic micro-organisms which include fungi and bacteria that antagonise plant pathogens undamaged. These antagonists prevent the soil from being recolonised by any pathogens that persist in local pockets. This is an example of microbial biocontrol even though it may also involve the use of chemicals. Strictly speaking this should be termed integrated control, i.e. practices in which more than one control measure are operating. In the above example there is a primary and direct effect of heat or chemicals on the plant pathogens and a secondary, indirect biological control effect operating through the activities of micro-organisms.

Even plant breeding can be included under the broadest definition of biological control especially if the new cultivar is resistant to disease because it supports a population of

controlling organisms. For example, Canadian spring wheat can be made resistant or susceptible to the disease commonly termed root rot by substitution of the chromosome pair 5B from a resistant or susceptible parent cultivar. This is correlated with changes in the root microflora although there may be no causal relationship (Deacon, 1983).

Some types of control must be excluded from even the broadest definition of biocontrol though they involve micro-organisms. Protection by attenuated strains of viruses against virulent ones, where some viral component (eg., surface protein) that is recognised and induces resistance in the plant which could be applied in the absence of the virus to give the same effect. A similar case would be if a product of a micro-organism (eg., antibiotic) was solely responsible for control and manufactured for this specific purpose. The use of ***Bacillus thuringiensis*** to control larvae of the Lepidoptera employs both organism and metabolite. The bacterium is grown in culture and produces a mixture of spores and toxin crystals which together constitute the control agent. In many cases the crystals alone can be used to give control, but the bacterium is recognised to play a secondary role in the control process by invading hosts previously weakened by the toxin. One of the reasons given for including plant breeding as a method of biological control is that in the foreseeable future it may be possible to incorporate microbial genes into the plant genome to achieve control eg., in the control of crown gall disease.

In summary microbial biocontrol can be achieved via several different mechanisms but all have one thing in common: an integral part of the control process is the activity of a micro-organism and biocontrol can therefore be considered as a practical application of a microbial ecological process.

1.4 Biological control in agriculture

The major pests confronting man are insects, weeds and plant pathogens. Insects ravage crops and transmit disease organisms of man, his stock and his crops. Weeds compete with crops and choke and poison waterways, as well as harbouring harmful pests and pathogens. Plant diseases caused by fungi, bacteria, viruses, mycoplasmas, nematodes and higher plants lead to severe losses as a result of the continuous impairment of crop

physiology. General texts on pests and diseases have been written by Fox Wilson (1960), Gram ***et al.***, (1969), Stapely and Gayner (1969), Fletcher (1974), Jones and Jones (1974) and Kranz ***et al.***, (1979) (as cited in Burge, 1988).

Though extensively studied by many scientists with notable progress being made, the ecology and complex interactions between soil micro-organisms remain somewhat of an anomaly. In the course of growing millions of acres of crops during thousands of years of agricultural development mankind has however found many examples of the effects of microbial interactions.

Biological control is based on microbial interactions and involves a study of the factors that regulate or affect the ecosystem. This is the basis for research on biological control in plant pathology, nematology, and entomology, and provides the basis upon which to build this inter-disciplinary science. Advances in molecular biology have also provided new tools to study the genetics of microbial interactions in biological control. A greater understanding of these interactions will lead to improvements in the selection and adaptation of biocontrol agents and thus will enhance pest and disease control.

Biological control of plant pathogens has evolved as an extension of research on soil-borne pathogens and on the ecology of the rich microbial flora found in the rhizosphere. This work began during the second half of the 20th century and now forms the basis for the study of biological control, based heavily on microbial and microbe-plant interactions. In this field of study, antagonism, competitive inhibition, and hyperparasitism are common mechanisms describing some of the interactions between control agent and target. Not all useful interactions involve microbes however, some small insects and other members of the soil fauna have also been seen to feed on and destroy plant pathogens (Nelson, 1989).

Man is increasingly aware of the environment being polluted by accumulation, in soil and water, of chemical residues that are harmful to himself; his crops and animals and to soil micro-organisms. This realisation has led him to consider biological systems in an

attempt to destroy pathogens without creating other disruptive side effects. Although difficult to achieve, biological control seems to be one of the most attractive alternatives to toxic agricultural biocides.

Biological control agents will be more specifically targeted and have a narrower or broader spectrum of activity than most chemical pesticides and successful use will therefore require more careful management. Many of the biocontrol agents that will be researched will unfortunately, through lack of commercial support for their development not be fully exploited. Research and development of biocontrols agents will therefore depend heavily on funding from governments and other non-profit organisations (Nelson, 1989).

All pests and disease organisms, may be subject to a degree of natural control. The fact that micro-organisms interact with each other, and may inhibit growth or cause death, has been known in culture for more than 100 years (reviewed in Baker, 1987 a). Actual control of pathogens in systems closer to the natural conditions was demonstrated by Hartley (1921), Henry (1931) and Millard and Taylor (1927) who used micro-organisms in mixtures or from pure cultures against various soil-borne diseases. The idea of using added inoculants in biological control was established almost 60 years ago. The ideas however remained unexplored during and after World War II with the enormous development of chemical pesticides. More recently however there has been an enormous increase in the amount of research effort devoted to biological control (Baker, 1987 b). Researchers have explored possibilities of using a variety of viruses, bacteria and fungi as biocontrol agents, although only a few have reached the status of commercial usage (Campbell, 1989 b).

Biological control of soil-borne pathogens by introduced micro-organisms has been studied for over 65 years (Baker, 1987 b; Cook and Baker, 1983), but during most of that time little commercial exploitation has occured. Since 1965, however, interest and research in this area have increased steadily (Baker, 1987 b), as reflected by the number of books (Baker and Cook, 1974; Chet, 1987; Cook and Baker, 1983; Papavizas, 1981)

and reviews on the topic (Baker, 1968; Brown, 1974; Burr and Caesar, 1984; Jatala, 1986; Moore, 1979; Papavizas and Lumsden, 1980; Schroth and Hancock, 1982; Suslow, 1982). Meanwhile there has also been a shift in opinion that biological control can have an important role in agriculture in the future, and it is encouraging that several companies like ICI plc, etc now have programmes to develop biocontrol (Weller, 1988). This is undoubtedly a response to public concern about hazards associated with chemical pesticides.

Attention has been diverted towards finding biocontrol agents for soil-borne pathogens rather than foliar pathogens. The main reason for this is that foliar pathogens are often adequately controlled by cheap, effective, chemical methods (usually fungicides) or through varietal resistance with which biological control would have to 'compete'. There are few fungicides effective against soil-borne diseases, especially in agricultural rather than horticultural applications, and breeding for resistance has been principally used to control root disease. Foliar diseases have however, been the first target for biocontrol systems because they can be relatively easily recognised and assessed in the field trials. Root diseases are nonetheless seen as a major constraint on crop productivity (Cook, 1987) and their biocontrol is therefore receiving more attention (Campbell, 1989 a).

In identifying antagonists against soil-borne pathogens, micro-organisms that can grow in the rhizosphere are ideal candidates, since the rhizosphere, provides the front-line defence for roots against attack by pathogens. Pathogens encounter antagonism from rhizosphere micro-organisms before and during primary infection and also during secondary spread on the root surface. In some soils described as microbiologically suppressive to pathogens, microbial antagonism of the pathogen is especially great, leading to substantial disease control (Schneider, 1982). Although pathogen suppressive soils are rare, those which have been identified are excellent examples of the full potential of biological control of soil-borne pathogens (Weller, 1988).

It is impossible to report all biocontrol agents that have been explored against insect, plant, microbial and other pests. Many potential viral, bacterial and fungal biocontrol agents have been looked into in the past years but few have reached the stage of commercial production. Table 1.1 and 1.2 gives an indication of the bacterial and fungal biocontrol agents that have reached the commercial market.

Antagonist	Pathogen and disease	Comment
Agrobacte--rium Strain 84	Agrobacterium tumefaciens crown gall in horticulture	Special interest in the Ti plasmid as a gene vector from bacterium to eukaryotic host. Antagonist used as a root or cutting dip. Operates by competition for adsorption sites and production of bacteriocin. Biocontrol commercially available (Clare et al., 1987)
Erwinia herbicola	Erwinia amylovora fire blight	Short period of control is needed during flowering. See also Pseudomonas Probably competition (Lindow, 1985)
Fusarium lateritium	Eutypa armeniacae apricot canker	Uses combined fungicide treatment and a fungicide tolerant biocontrol agent. Commercial (Carter, 1983)
Pseudomonas syringae	Erwinia amylovora fire blight	Used in conjunction with control of frost damage. Antibiotics and siderophores probably not important (Beer et al., 1984)
Pseudomonas spp., especially P.putida and P.fluores--cens	Many diseases, eg. take-all (Gaeumannomyces graminis), Pythium root rot, Rhizoctonia, Fusarium, etc., also for the control of frost damage	Fluorescent pseudomonads are one of the main groups of bacteria being developed for commercial use. None yet available Produce antibiotics, siderophores and possibly lytic enzymes. (Cook and Baker, 1983; Weller, 1988)

Table 1.1 - List of antagonists in, or near to, commercial use :
In " Microbial inoculants of crop plants" by Campbell (1989 b).

1.4.1 Fungal biocontrol agents

Fungi have evolved the ability to utilise a variety of substrates and colonise various habitats. During this evolution, the problem of competition from other organisms for food and/or space in the fungal habitat has resulted in development by certain fungi of methods of antagonism for the suppression of these competitors. Some fungi through competition for a substrate may reduce the impact of organisms that are indirectly harmful to man and thereby have the potential to act as biological control agents.

There has been a long history of efforts to deploy fungi to antagonise plant pathogens and thereby control diseases. It is not difficult to isolate fungi which are antagonistic to plant diseases, especially ***in vitro***. The difficulty arises in developing a successful disease biocontrol agent which will work in the field, and there have been remarkably few successes to date. Generally fungi have only been used as antagonists of fungal and not bacterial plant pathogens. Pests and disease organisms, with the possible exceptions of viruses, bacteria and mycoplasmas, may be subject to a degree of natural control by fungi, which are either predatory, parasitic or antagonistic.

A lot of work has been carried out to obtain fungal antagonists against pests other than plant pathogens (Lisansky and Hall, 1983). Attention will be devoted here to potential biocontrol agents of pests and plant pathogens that are, close to commercialisation. There is more interest in development of fungal antagonists against plant pathogens than any other pests, simply because of the huge economic losses that are faced as a result of plant diseases. In the United States alone, at least $4 billion is lost annually due to soil-borne fungal pathogens (Acuff, 1988). Current and future legislative regulation, especially in the US, to restrict pesticide (including fungicide) use will compound these conservatively estimated losses even further. Although restrictions are being imposed to protect food quality and the environment, chemicals are still the only means presently available to prevent diseases of food and fiber crops. In recent years, the need to develop disease control measures as alternatives to chemicals has become high priority for scientists worldwide. Biological control, especially using fungal antagonists against fungal plant pathogens, has therefore gained considerable attention and appears to be a

promising viable supplement or alternative to chemical control (Cook and Baker, 1983; Papavizas, 1981, 1985)

There are many reports of control of diseases in the research literature, however most of these control agents have not been commercially exploited. This may be, due to failure of the organism to achieve control especially in the field. There are also a number of potentially useful antagonists which have not been, and will not be, developed, despite their success, since the controlled crop or disease may not be important enough to warrant commercial development. Another constraint is that research information may have been published, thereby preventing patent protection without which commercial investment is doubtful.

Among fungal antagonists that have been researched ***Trichoderma*** spp. have proved to be the most successful. This competitiveness may be illustrated by their widespread occurrence and other specialised qualities that are discussed below that makes them unique.

1.5 TRICHODERMA

Trichoderma are soil fungi that occur ubiquitously, first described by Persoon in 1794 as a conidiating (asexually reproducing) fungal species. The potential use of ***Trichoderma*** species as biocontrol agents was first suggested more than 50 years ago by Weindling (1932) who demonstrated the parasitic activity of members of this fungus genus to the pathogen such as ***Rhizoctonia solani.***

1.5.1 Taxonomy

The similarity between the anamorph (asexual reproductive state) of ***Trichoderma viride*** Pers.ex.S.F. Gray and the teleomorph (sexual) ***Hypocrea rufa*** (Pers.ex Fr.) Fr. was noted by the Tulasne brothers in 1865 and caused considerable confusion regarding the taxonomic status of ***Trichoderma***. The classification of ***Trichoderma*** spp. has evolved slowly, however, more recent efforts by Webster (1964), Doi (1979) and Rifai

(1969) have helped to clarify this taxanomic problem. Webster (1964) initially showed the distinction between ***H.rufa*** and ***H.gelatinosa*** by indicating their respective anamorphs to be ***Trichoderma*** and ***Gliocladium***, while Doi over the last 20 years, has devoted studies to clarifying further distinctions between such teleomorphs and anamorphs (Doi and Doi, 1979). In noting the complexities of morphologic speciation, Rifai (1969) presented a classification guide to the genus ***Trichoderma*** based on nine "species aggregates". This has been widely accepted as a practical guide, with incorporations of further species by Domsch ***et al.***, 1980; and Doi and Doi, 1979, while retaining the "species aggregate" concept.

1.5.2 Morphology

Trichoderma colonies grow rapidly, initially forming a smooth white surface and becoming compact following conidiation. Colonies may produce yellow pigments, but mature colonies are green from the colour of the conidial masses. Conidia form on branched aerial conidiophores, speciation being based in part on conidiophore morphology (Rifai, 1969). Conidia (phialospores) are produced on inside flask-shaped phialides and are released at the tip to form clusters, that is, basipetal succession via production of enteroblastic conidia. The initial phialide cell wall is ruptured on release of the first conidium; and as successive conidia are released, a ridged collarette comprised of rings of residual cell wall material builds up inside the rim of the phialide tip (Hammill, 1974, as cited in Eveleigh, 1984). The conidia lack distinctive characteristic apart from those of ***T.viride***, which have rough walls (Eveleigh, 1984).

1.5.3 Sporulation and Germination

Most species of ***Trichoderma*** are photosensitive, sporulating readily on many natural and artificial substrates in a concentric pattern of alternating rings in response to diurnal alternation of light and darkness, with conidia being produced in the presence of light. The photo-induced conidiation in ***Trichoderma*** can be inhibited by chemicals such as azaguanine, 5-fluorouracil, actinomycin D, cycloheximide, phenethyl alcohol, and

ethidium bromide. Though light and various chemicals play a role in sporulation; the molecular and biochemical processes involved in germination have largely been ignored due to the ease with which conidia of ***Trichoderma*** germinate on many substrates (Papavizas, 1985).

1.5.4 Ecology

Trichoderma spp. comprise a group of fast-growing Hyphomycetes that are extremely common in agriculture, prairie, forest, salt marsh, and desert soils in all climatic zones (Danielson and Davey, 1973 a; Domsch ***et al.***, 1980). They are particularly prevalent in the litter of humid, mixed hardwood forests, comprising a minor component of the microbiota in the initial colonisation but subsequently becoming more dominant. It has been noted that there is correlation between species distribution and a variety of factors (Papavizas, 1985). ***Trichoderma polysporum*** and ***T.viride*** occur in cool temperature regions, while ***T.harzianum*** is characteristic of warm climates. This correlates with optimal temperature requirements for each species (Danielson and Davey, 1973 b). In general, ***Trichoderma*** species appear to be more prevalent in acidic soils, and Gochenaur (1970) (as cited in Papavizas, 1985) was able to correlate the occurrence of ***T.viride*** with acid soils from cooler regions in Peru.

The widespread occurrence and effective colonisation potential of ***Trichoderma*** species can be associated with several factors including their metabolic versatility; resistance to microbial inhibitors; and their antagonism to other microbes. Germination of ***Trichoderma*** spores can also be relatively insensitive to fungistasis (Emmatty and Green, 1966, as cited in Papavizas, 1985). They are relatively resistant to synthetic chemicals such as carbon disulphide (Bliss, 1951; Webster, 1964), captan and chloropicrin (Anderson, 1962), formalin (Warcup, 1951, as cited in Papavizas, 1984), dichloropropane-di-chloropropylene (D-D) (Altson, 1950), and allyl alcohol, methyl bromide, and Semesan (2-chloro-4-hydroxy-mercuriphenol) (Woodcock, 1971). The dominance of ***Trichoderma*** species in soil following fumigation is well known and is probably due to their inherent resistance to fumigants and their enhanced ability to

colonise in the absence of competitive micro-organisms. This postfumigation dominance is of special significance in the use of ***Trichoderma*** as a biological control agent (Bliss, 1951). This remarkably broad-based tolerance of ***Trichoderma*** to growth inhibitors of both microbial and abiotic origin, presumably facilitates their effective colonisation of soil (Papavizas, 1985).

1.5.5 Nutrition and physiology

Trichoderma species are metabolically versatile and can utilise a diverse range of subtrates. Carbon sources include many sugars and polysaccharides such as cellulose, chitin, laminarin, pectin, starch and xylan (Danielson and Davey, 1973 a; Domsch ***et al.***, 1980). Considerable variation among species occurs in the utilisation of inulin, melezitose, raffinose, sucrose and tannic and gallic acids (Danielson and Davey, 1973 a). Rhamnose, ***i***-inositol and α-methyl-D-glucoside are exceptions and are poorly used.

It is somewhat enigmatic that in spite of the marked cellulolytic nature of most ***Trichoderma*** species, their ability to degrade wood (lignocellulose) is relatively weak. Strains can colonise wood by using nonstructural carbohydrates. They attack loblolly pine logs but, in doing so, destroy only the ray parenchymatous cells and the half-bordered pits (Hulme and Stranks, 1970). These actions can be put to good use to promote enhanced penetration and thus improved application of timbers preservatives (Johnson and Gjovik, 1970). However the attack is species specific, and no action is shown toward Douglas fir. In pure culture studies, several ***Trichoderma*** species were relatively ineffective in degrading dogwood leaves and loblolly pine needles (Danielson and Davey, 1973 a), beechwood (Butcher, 1968), and birch and pine blocks (Bergman and Nilsson, 1971).

As well as a variety of polymeric materials, ***Trichoderma*** species are one of the few groups of organisms that can metabolise C_1 compounds. ***T.lignorum*** can metabolise methanol, but grows very slowly (0.029 generation/h). ***Trichoderma*** strains can also degrade hydrocarbons (Davies and Westlake, 1979, cited in Eveleigh, 1984), and are

major components of populations from soils polluted with oil (Pinholt *et al.*, 1979, as cited in Eveleigh, 1984). Many nitrogen sources (ammonium compounds, L-alanine, L-aspartate, l-glutamic acid, and proteins) are all readily utilised, although nitrate assimilation is often poor and is species dependent (Danielson and Davey, 1973 a). From the above nutritional patterns, Okuda *et al.*, (1982) suggest that the utilisation of sucrose, raffinose, melezitose, and nitrate, and their reactions toward tannic and gallic acids, can be used as an aid in classification.

Trichoderma species are excellent producers of enzymes. Cellulase has received particular attention in the utilisation of biomass as a source of feedstock chemicals (Bungay, 1981). ***Trichoderma*** cellulase is noteworthy in that it attacks crystalline cellulose through the synergistic action of three major types of enzymes: cellobiohydrolase, endoglucanase and cellobiases. Cellulase is generally induced by cellulose and also by lactose and sophorose, this latter feature not being commonly found in fungi. Through the use of hypercellulolytic mutants and controlled fermentation conditions, extraordinarily high yields of cellulase (2% extracellular proteins, 70% of which is cellulase) have been obtained. Scale-up to 150 liters has also produced good yields (Watson and Anziska, 1983). Which is probably related to the enhanced amounts of endoplasmic reticulum, at least in the mutant ***T.reesei*** RUT-C30.

With the ability to utilise such a variety of substrates and, in combination with being able to survive under relatively adverse conditions, it is not too surprising that ***Trichoderma*** spp. are seen to be general spoilage organisms. They have been found in microcosms associated with the deterioration of paintings, masonry, rubber, plasticizers, polythenes (Pitt, 1981; Rose, 1981) and jet fuel (Sheridan and Soteros, 1974).

1.5.6 Metabolite production

Weindling (1934, 1937 and 1941) was the first to show the production of an antifungal metabolite by a species of ***Trichoderma*** i.e., ***T.lignorum*** however this fungus was later stated to be ***Gliocladium fibriatum***). Weindling and Emerson (1936) isolated in

stated to be *Gliocladium fibriatum*). Weindling and Emerson (1936) isolated in crystalline form an organic metabolite very toxic at high dilution to ***Rhizoctonia solani***. The metabolite was later given the name of gliotoxin. Brian and McGowan (1945) described a second highly fungistatic antibiotic, viridin, produced by ***T.viride***. Webster and Lomas, 1964 eventually questioned whether the gliotoxin and viridin were produced by ***Trichoderma***. Re-examination of the cultures from which these toxins were obtained revealed that the ***T.viride*** (***Hypocrea rufa***) actually matched the type isolate of ***Gliocladium virens*** (***H.gelatinosa***) (Papavizas, 1985).

Almost thirty years after the discovery of gliotoxin and viridin, interest has been renewed in toxic metabolites produced by species of ***Gliocladium*** and ***Trichoderma*** and in the role they may play in biocontrol. Howell and Stipanovic, 1983 reported on the isolation and structure of a new toxic metabolite from ***G.virens***, a diketopiperazine (Stipanovic and Howell, 1982) given the trivial name of gliovirin, that is active against ***Pythium*** spp.

Better fermentation and characterisation of ***Trichoderma*** metabolites has increased interest in this area. Dennis and Webster, 1971 (a) showed that ***Trichoderma*** spp. produce antibiotics different from gliotoxin and viridin. However, other chloroform soluble antibiotics were produced such as trichodermin (by ***T.viride*** and ***T.polysporum***) and other peptide antibiotics by ***T.hamatum***.

Trichoderma spp. are not only good sources of various toxic metabolites and antibiotics, but also of various enzymes such as chitinase, exo- and endoglucanases, and cellobiase (Papavizas, 1985).

1.5.7 Biocontrol applications todate

The antagonistic ability of ***Trichoderma*** was discovered more than 50 years ago (Weindling, 1932) and the potential of the fungus to serve as a biocontrol agent was already suggested at that early stage. However, only during the last few years has there been a worldwide effort in developing the fungus for commercial use.

Trichoderma has been noted to be antagonistic to several fungi (Chet, 1987). Before 1970 much of the work on biocontrol involved indirect enhancement of indigenous ***Trichoderma*** population by manipulating the physical conditions within the environment. It has been known that ***Trichoderma*** occupy soil that has undergone harsh chemical treatments, but the implications of this for biological control is still open to debate. A chronological representation of the use of ***Trichoderma*** against various pathogens through the years and its current commercial availability are presented in Table 1.2.

Antagonist	Pathogen and disease	Comment
T.viride	Heterobasidium lignosus and noxius	Altson, 1950 Soil inoculation of antagonist following treatment with D-D (Dichloropropane-dichloropropylene)
	Heterobasidium annosum root rot of pine in acid soils	Rishbeth, 1951, 1975 Applied on to cut stem
	Armillaria mellea causes serious root rots in trees and plants	Bliss, 1951 Carbon disulphide fumigation of the soil resulted in dominance of Trichoderma as they are resistant to fumigation and reduced population of pathogens
	Chondrostereum purpureum Silver leaf disease of fruit trees	Grosclaude, 1970 Applied conidia of Trichoderma to wounds during cutting by means of special pruning shears **Antagonists inoculated into stem and reduces existing symptoms and future infection. Commercial trials** (Corke and Rishbeth, 1981)
	Verticillium dahliae Root rot of strawberries	Jordan and Tarr, 1978 Dipped roots of strawberry runners into suspension of Trichoderma

T.harzianum	Sclerotium rolfsii Various rots of of stems, bulbs etc.	Wells, et al., 1972 was the first to report large-scale use of Trichoderma preparation on solid media (ground annual ryegrass seed) for field use.
	S.cepivorum White rot of onions	Abd-El Moity, et al., 1981, 1982 and Papavizas, 1981 - used Trichoderma on solid substrate
	Rhizoctonia solani Fruit rot of cucumber	Lewis and Papavizas, 1980
		Trichoderma is now applied as seed coat or to growing medium against various diseases caused by the above mentioned pathogens (especially to horticultural crops) (Papavizas, 1985)
	V.dahliae cucumber and cotton wilt	Fedorinchik, et al., 1975 also used Trichoderma on solid substrate
T.hamatum	Pythium and Phytophthora etc., damping-off of seedlings	Chet and Baker, 1980 - recognised its use as a biocontrol agent Harmen, et al., 1980, 1981 - used as a seed treatment
		At present the antagonist are used as a seed coat or pellet mixed with sterile compost, commercially available. Possibly with fungicide and a fungicide resistant antagonist. Produces antibiotics, mycoparasite (Chet, 1987; Papavizas, 1985)

Table 1.2 : Trichoderma species exploited as biocontrol agents in early years and the commercial availability of some isolates for certain plant pathogens at present (highlighted).

1.5.8 Integrated control with *Trichoderma*

The use of ***Trichoderma*** in combination with various chemicals can be achieved by either: 1) application of the fungus simultaneously with a sublethal dose of the chemical, 2) introduction of the fungus immediately after soil fumigation or solarisation to prolong the effects of these treatments.

Curl *et al.*, (1977) found, that small doses of pentachloronitrobenzene (PCNB) together with ***Trichoderma*** spp. (1g and 10g/g of soil respectively) in sterile soil were slightly more efficient against ***Rhizoctonia solani*** than the biocontrol agent alone. Davet *et al.*, (1981) showed that ***Trichoderma*** spp. are sensitive to benomyl, but Ahmad and Baker (1987) produced a benomyl-tolerant mutant of ***T.harzianum***, which was rhizosphere-competent when benomyl was added at 10ug per gram of soil. The mutant produced by (Ahmad and Baker, 1987) has a significant advantage over the wild-type fungus since it can be applied with benomyl, or in fields previously treated with benomyl (Chet, 1990 a).

Disease control with a combination of ***Trichoderma*** and solar heating of the soil was developed in Israel (Katan *et al.*, 1976 a, b) about 12 years ago as a new approach to controlling diseases caused by soil-borne pathogens. An isolate of ***T.harzianum***, capable of attacking both ***R.solani*** and ***Sclerotium rolfsii*** and applied after solarisation as wheat-bran preparation improved control of diseases caused by these fungi on potatoes. The combination of ***T.harzianum*** and solar heating of the soil reduced the inoculum density of ***R.solani*** and retarded its subsequent build-up in both field plots and under greenhouse conditions. Neither ***Trichoderma*** nor solar heating alone gave as good control as the combination treatment (Elad, *et al.*, 1980). Lifshitz *et al.*, (1984) have since shown that sublethal heat-treatment causes cracks in the sclerotial rind of ***S.rolfsii***, thereby enabling heat-tolerant fungi such as ***Aspergillus fumigatus*** to attack the sclerotia, reduce the inoculum density of the plant pathogen, and lower the incidence of diseased plants (Chet, 1990 a).

1.5.9 Antagonistic mechanisms

A wide range of antagonistic mechanisms can be exhibited by fungi ***in vitro*** and ***in vivo***. Such antagonism can occur by the production of volatile and non-volatile antibiotic or cell wall-degrading enzymes. However the ecological success of the antagonist can also be governed by its ability to colonise and utilise substrates at the target site thereby, allowing it to compete more effectively. Such actions of antagonists on pathogens do not necessarily occur independently of one another and successful antagonists may

exhibit more than one mechanism. ***Trichoderma*** species are an excellent example of successful fungal antagonists. Their widespread occurrence and effective colonisation potential can be associated with several factors, including their metabolic versatility, their resistance to microbial inhibitors, and their antagonism to other microbes (Lynch, 1990). Antagonistic mechanisms include - 1) competition for nutrients; 2) mycoparasitism via lytic enzyme production; 3) inhibition by volatile and non-volatile antibiotic products; 4) production of "siderophores" that may have a role to play in the antagonistic mechanism

A detailed review of each of these individual mechanisms is included in the introduction to corresponding chapters of the thesis.

It is clear that the potential of ***Trichoderma*** as a biocontrol agent has been explored thoroughly to date for the purpose of disease control in agriculture. This valuable knowledge however, can be extrapolated to analyse the potential of these organisms as antagonists of wood decay fungi. There are still many unanswered questions in the agricultural area and to extrapolate from studies in soil to another complex ecosystem, wood is certainly a challenge.

1.6 Wood

Wood has served mankind since prehistoric times, and has contributed to his survival and to the development of civilization. Moreover, wood continues to be the raw material for a large number of products even in modern times, although other competitive materials (metals, cement, plastics) are now available. The value of wood however is preserved in many traditional uses, and grows steadily with its use in new products to meet the increasing needs of man.

After harvesting in the forest, wood is converted into a great number of products by sawing, slicing, gluing, chipping, pulping, modification by impregnation with chemicals, or chemical processing. In chemical processing, the change is so drastic that

wood origin cannot be recognised. Products of primary industrial processing include poles, posts, lumber, laminated wood, veneer, plywood, particleboard, fiberboard, pulp and paper and, in turn, these are made into products for final use (furniture, etc.). Products of chemical processing include synthetic fibers, photographic films, explosives, chemicals, and many others (Tsoumis, 1991).

Wood is also an important fuel material for cooking, heating, and production of steam, which may be utilised as a source of energy. About half of the world's production of wood is used as fuel. With the existing energy problems, wood, as a renewable product of nature, is acquiring renewed interest as fuel.

These multiple services are due to certain advantages: wood is aesthetically unrivaled as a material, because it is available in a great variety of colours, textures, and grains. It also gives a feeling of "warmth" to touch and sight, which is not possessed by competitive materials; it is very strong mechanically in relation to its weight; insulating to heat and electricity, exhibits little thermal contraction and expansion, and has good acoustical properties (utilised in making musical instruments). It does not oxidise (rust) and shows considerable resistance to mild concentrations of acids; may be easily machined and can be easily bonded by nailing with metal connectors and gluing. Wood is the main source of cellulose which is the basis of numerous products. It is found in most parts of the world and is a renewable resource - in contrast to petroleum, metal ores, and coal, which are gradually but steadily exhausted; and importantly is biodegradable.

Wood however also has its disadvantages: it is hygroscopic - holds moisture in contact with liquid water or water vapour and the gain or loss of moisture, within certain limits, results in dimensional changes. It is an anisotropic material - presents differential mechanical strength and differential dimensional changes in different structural directions. It may burn and decay and has variable structure and properties. Because it is a product of biological processes its production is influenced by environmental factors

and heredity. As with any other material a sound knowledge of its advantages and disadvantages is prerequisite to the rational utilisation of wood (Tsoumis, 1991).

Wood has been used by man for thousands of years as a cheap and readily available raw material with many structural and decorative functions. Wood is no longer cheap however, and Britain now imports more than 7 billion pounds of wood and wood products annually (Anonymous, 1990). It is also no longer plentiful and forestry management practice in the technically developed world has resulted in considerable control of deforestation and decreased exports to the major wood importing nations.

Trees are classified as hardwoods or softwoods. Hardwood trees are dicotyledonous, have broad leaves which are frequently are shed at the end of each growing season and produce seeds in closed seed cases. The timber of hardwoods has relatively large-diameter vessels which form the major arteries for sap conduction. Softwood trees, or conifers, have needle-like or scale-like leaves which are also shed annually in some species, but generally these are held longer than a single growing season and the trees are known as "evergreens". The wood of softwood species does not have large sap-conducting arteries, this function being performed by elongated cells called longitudinal tracheids.

Softwood trees evolved earlier than the hardwoods. There are more than 500 species of softwoods in the world, most of which are found in the Northern Hemisphere. Hardwood species, however, number in the tens of thousands with over 110 different species of timber available in the United Kingdom alone. The timber species that originate from the UK are three softwood species Douglas fir, Scots pine and Sitka spruce all of which are used for a variety of purposes (Annonymous, 1991).

1.6.1 Wood structure and composition

Wood represents the annual accumulation of xylem cells originating from a lateral meristem or cambium. The cambium is a continuous ring of meristematic cells that form around the outer circumference of the developing stem. The cambial cells divide

periclinally and form xylem cells (wood) to the inside and phloem cells (bark) to the outside. There are two general types of cambial cells, based on shape and the tissues formed. Vertically elongated cambial cells, form the longitudinal parenchyma and the tracheids in the conifers; and the vessels and fibers in hardwoods. Horizontally elongated or cuboid cells, form the radially aligned wood rays, consisting of ray parenchyma and, in conifers, also ray tracheids.

The vascular cambium divides only during the growing season. In temperate zones, the annual accumulations of xylem often result in abrupt annual rings, usually consisting of a zone of rapid growth (earlywood) in the spring and early summer and slower growth (latewood) later in the growing season. In tropical zones, annual rings are not so apparent in many species, and the differences in growth accumulations probably reflect regular seasonal patterns in rainfall. Annual ring elements in conifers consist primarily of tracheids in uniform radial rows.

The outer zone of the stem, which contains many living parenchyma cells, is the sapwood. Sapwood is white in colour in most species and functions for conduction, food storage and stem protection. The pith is a small zone at the centre of the stem, consisting of parenchyma cells and originating as a primary tissue from the ground meristem. As the girth of the tree expands and the inner sapwood tissues age and recede from the phloem, increasing numbers of parenchyma cells slowly die, and the tissue develops into heartwood. Significant chemical and structural changes during the transformation of sapwood into heartwood include the loss of starch, the deposition of extractives, and the aspiration of the pits in conifers or the formation of tyloses in hardwoods. These changes may render the wood more resistant to biological attack or decrease the permeability, making the preservative treatment more difficult (Desch, 1973; Wilcox, 1973).

The structure of softwoods is less complex than that of hardwoods, which have more cell types. Tracheids make up the majority of the longitudinal elements of softwoods and function both as conductive and strength providing tissues. As a result most

microscopical effects of degradation by micro-organisms in softwoods will be observed in the tracheids and it is decomposition of these elements, particularly the thick-walled latewood tracheids, that most greatly affects mechanical properties of the wood. A small proportion of the longitudinal elements of some softwoods consists of parenchyma cells, either distributed among the tracheids or aggregated to form the walls of resin ducts. These elements (resin ducts) may be important in the degradation process because they provide ready avenues for longitudinal distribution or because they contain storage materials and possess thin cell walls (longitudinal parenchyma). Other softwood structures of importance in wood degradation are the rays, which provide avenues of transverse distribution and often contain large quantities of storage materials. Rays may consist of both parenchyma and tracheid cell types. Removal of storage material from rays, or destruction of ray cell walls, may have profound effects upon transverse movement of liquids within the wood (Wilcox, 1973).

The tissue functions in hardwoods are the same as in softwoods, but are carried out by a large number of specialised cell types. The bulk of the cell-wall material in most hardwoods is contained in fibers having relatively small diameters, narrow lumina, and thick walls. Like latewood tracheids in softwoods, fibers in hardwoods are the elements in which the effects of degradation by micro-organisms are most often conspicuous and have the greatest effects upon mechanical properties of the wood.

Although the various types of wood elements differ widely with regard to size and shape, they share some similarity with regard to cell-wall ultrastructure This is particularly true of softwood tracheids and hardwood fibers. A typical cell wall of a tracheid or fiber consists of the following layers: middle lamella(ML); primary wall(P); and secondary wall consisting of an outer (S1), middle (S2), and inner (S3) layer, and in some cases a warty layer or tertiary lamella on the lumen surface of the S3 (Figure 1.1).

The inner cell cavity termed the cell lumen is an inert space occupied by air and/or water. The lumen volume, collectively, in most woods is large, and is the critical cell-wall zone where most decay fungi initiate the decay process. Another principal cell-wall

zone initially penetrated by hyphae during wood colonisation and degradation by the stain and decay fungi are the pits. Pits (softwoods) are gaps in the secondary wall containing a modified portion of the adjacent primary walls called the pit membranes. Conduction of water and various solutes between adjacent cells occurs through the pits. The types of pits vary with cell types and plant species (Goldstein, 1977).

Wood cell walls consist primarily of cellulose, hemicellulose and lignin. In the cell wall, cellulose levels are highest in the secondary wall, hemicelluloses levels are highest in the S1 and lignin is present at highest levels in the middle lamella and primary wall. Cellulose is a long, linear polymer consisting of B-D-glucose units with (1-4) glycosidic linkages, and forms the cell-wall framework. Cellulose occurs in plant-cell wall as bundles of parallel-aligned molecules termed microfibrils. The microfibrils contain alternating crystalline and noncrystalline or amorphous zones. These two regions are known to display different degrees of resistance to enzymatic attack. The orientation of the microfibrils within the cell walls is also known to influence some types of microbiological degradation (Wilcox, 1973). The primary wall (PW) consists of a loose network of mostly axially oriented cellulose microfibrils. The S1 and S3 are narrow zones in the cell wall in which the cellulose microfibrils are arranged in a flat helix. The S2, which composes the bulk of the cell wall, consists of microfibrils arranged in a steep helix, oriented nearly parallel to the longitudinal axis of the cell. The microfibril orientations in the cell wall layers are illustrated in Figure 1.1. The S2 layer is the most important zone of the cell wall and is responsible for a majority of wood-strength properties, particularly its remarkable tensile strength.

The hemicelluloses are shorter, liner molecules containing hexose or pentose sugars as the monomer units. The monomer units in the main chain are, as in cellulose, connected by (1-4) glycosidic linkages. Some hemicelluloses are branched, and most contain side chains. The hemicelluloses are deposited around the microfibrils and form the cell-wall matrix. Lignin is an aromatic polymer formed by free radical polymerisation of three types of cinnamyl alcohols. This constituent is a huge amorphous polymer without a regular structure and forms an interpenetrating polymer system around and between the

hemicellulose-coated microfibrils of cellulose. Lignin also provides stiffness and strength, and is a very durable material and acts as a barrier against microbial attack of the more vulnerable carbohydrates in the cell wall.

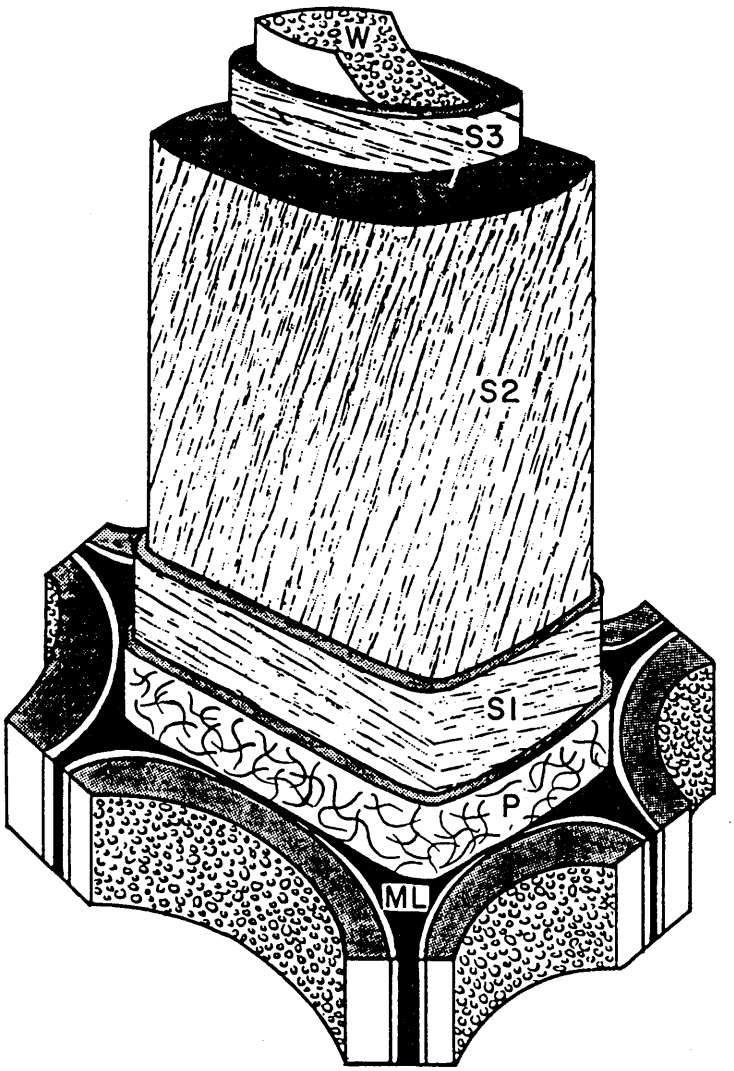


Figure 1.1 - Diagram of a wood cell showing the organisation and microfibrillar orientation of the major cell-wall layers. The layers are identified from the middle lamella (ML) and inward as primary wall (P), the S_1, S_2, and S_3 composing the secondary wall, and the warty (W) lining of the lumen surface (modified figure from Zabel and Morrell, 1992).

1.6.2 Wood decomposition : A target for biological control

Wood is readily decomposed and recycled in the forest ecosystem by various biotic and abiotic agents, however it is a very durable organic material when properly used and maintained. The major agents and types of wood decomposition can be grouped under abiotic and biotic categories and the major types of wood damage and their descriptions are as listed in Table 1.3.

Wood can be decomposed by micro-organisms either in the standing form in the forest or after it is felled. When it is in the form of a living tree the organisms involved are parasites causing disease and this aspect of microbial decomposition is more properly considered as forest pathology. From the viewpoint of the wood user, the important organisms are generally those which colonize the wood after it is felled. The colonisation sequence of wood by micro-organisms and the development of decay will be affected by differences in the type of wood (softwood, hardwood, heartwood and sapwood) as discussed in the case of commercial timbers by Dickinson, 1982. Structural and chemical differences constitute part of the ecological factors that will determine the process of decay. Other factors include: the rate of growth of the wood when it was being formed in the tree; the orientation of the grain of the wood with respect to the source of invading organisms and water; the moisture content of the wood translocating the soluble nutrients as it evaporates causing its accumulation; pH and temperature. Such factors give rise to a series of ecological niches which may be filled by a range of micro-organisms depending upon the particular circumstances (Levy, 1982).

In this thesis micro-organisms associated only with decay of wood in contact with the ground and their sequence of colonisation will be considered. A wide range of micro-organisms from many diverse taxonomic groups are readily available to colonise and destroy wood in contact with the ground. Some are specific to a particular wood under certain precise conditions of exposure. Others are more opportunist and may play a different role in the decay process under varying conditions. It is not possible to define or restrict each decay type within taxonomic frontiers,

Table 1.3 - Major types of wood damage and their description (table from Zabel and Morrell, 1992)

Type of damage	Causal agent(s)	General descriptions	Prevention or control
Weathering	Ultraviolet light, oxidation, swelling and shrinkage, leaching, and fungi	Unprotected surfaces develop a gray color and roughened texture	Ultraviolet light-resistant coatings
Thermal decomposition	High temperature	<200°C, uniform surface brittleness >200°C, charcoal in absence of oxygen, combustion around 275°C	Fire-retardant chemicals
Chemical decomposition	Caustic chemicals	With acids wood turns brown, chars, and becomes brittle; with bases wood bleaches and defibrillates	Chemically resistant woods
Mechanical damage	Mechanical forces rupturing surface tissues	Selective surface erosion in heavy friction zones	High-specific-gravity woods, edge grain, or chemically hardened woods
Insect damage	Termites	Localized honeycomb cavities, wood soiled and filled with frass	Insecticides or keep wood dry
	Borers	Tunnels, cavities, pinholes	
	Ants	Localized honeycomb cavities, wood channels clean	
Marine borer damage	Shipworms	Interior tunnels with lime-coated walls	Protective surface barriers or use wood preservatives
	Pholads	Large interior tunnels—near surface	
	Gribbles	Surface tunneling in tidal zone	
Decay	Fungi	*White* fibrous pockets or punky texture. *Brown* fibrous pockets or cubical checking pattern. *Soft* surface embrittlement and exfoliation in small fragments	Keep wood dry or use wood preservatives
Molds	Fungi	Colored spores or mycelium on the wood surface	Dry wood or use protective chemicals
Stains	Fungi	Sapwood discolored gray, black, brown, blue and intensified in ray parenchyma	Dry wood or use protective chemicals
Ray cell and cell-wall damage	Bacteria	Soft surfaces, ray cells destroyed, microscopic tunnels in cell walls	Keep wood dry or use wood preservatives

however since the same organism may, given suitable environmental conditions, fill one or several ecological niches or physiological groups (Clubbe, 1978).

Studies on the micro-organisms colonising wood and on the sequence of events leading to the onset of decay have shown the importance of classifying the species involved, or the basis of their effect on the wood. Six such groupings were recognised by Clubbe (1980), based on his own observations and other studies published in the literature. They comprised: Bacteria, Primary moulds, Stainers, Soft rots, Basidiomycetes (including white and brown rots), and Secondary moulds. The damage caused by each of these organisms in their order of colonisation of ground contact wood is as discussed below.

a) Bacteria - This group includes gram-negative aerobic rods (eg., ***Pseudomonas***) or facultatively anaerobic rods (eg., ***Erwinia nimipressuralis***), as well as endospore-forming rods and cocci (***Bacillaceae*** family) some of which are obligate anaerobic. It also includes actinomycetes such as ***Streptomyces*** species. Knuth (1964) in a comprehensive exploratory survey of bacteria in wood products, traced 198 isolates of ***Bacillus, Aerobacter*** and ***Pseudomonas*** - all common soil and water inhabitants. These micro-organisms are usually the primary colonisers. The attack of the wood cells however proceeds very slowly, as bacteria cannot grow in size, their expansion being determined by cell division. Invasion is primarily through the ray parenchyma cells although random distribution is also observed and proliferation results in increasing porosity of the wood cells thereby allowing further colonisation. Unless conditions are ideal for growth bacteria do not generally, produce significant decay in timber. However, over very long periods they are known to destroy pure cellulose and are also capable of causing serious damage to lignified tissues (Nilsson and Daniel, 1983) The factor most conducive to bacterial damage of wood is high moisture content. Since the same condition often limits fungal growth and allows bacteria to decay wood that is waterlogged. Bacteria are prevalent even in deeply submerged, water-saturated wood which denotes a special ability of the organisms to develop with extremely little or no oxygen (Knuth and McCoy, 1962).

Greaves (1971) placed the bacteria that colonise wood into four categories: bacteria that utilise the cell contents of rays and affect the permeability of wood to liquids but do not alter the strength; bacteria that attack the cell walls directly; those that are associated with other micro-organisms in wood and contribute to the decomposition process and antagonistic bacteria that are inhibitory to other micro-organisms that colonise wood. It has been noted that damage to the pit membrane may create passageways from cell to cell and in doing so may condition the wood to favour invasion by decay fungi. The bacteria also fix atmospheric nitrogen which provide an additional source of nitrogen that may enhance fungal growth (Levy ***et al.***, 1974; Baines and Millbank, 1976). Bacteria also directly facilitate radial penetration by opening up the wood rays and the horizontal resin ducts in softwoods. Prolonged infestation can result in serious loss in strength of wood (Eriksson, ***et al.***, 1990).

Bacteria as a class may be as remarkable as some of the soft rotters and moulds due to their tolerance of toxic chemicals. Besides their ability to detoxify creosote, bacteria have shown a high tolerance of copper-chrome-arsenic, pentachlorophenol and tributlytin oxide (Scheffer, 1973). Recently it has been shown that bacteria that were isolated from freshly felled, anti-sapstain treated timber could detoxify methylene bisthiocyanate (MBT) (Wallace ***et al.***, 1993).

b) Primary moulds - These organisms comprise the first fungal colonists and do not appear to be capable of degrading cellulose or lignin. They thrive on the sugars or simple carbohydrates present in the ray parenchyma of the sapwood or derived from the soil. Phycomycetes, Ascomycetes, and Deuteromycetes are all represented in this group. Most troublesome of the wood moulds are species of ***Trichoderma, Gliocladium***, ***Penicillium Alternaria*** and ***Aspergillus***. These moulds are airborne, opportunistic fungi with hyphae that are normally colourless, but discolour the wood by forming masses of pigmented spores on the wood surfaces. They discolour surfaces and wood chips for pulping with their coloured spores, and also cause a discolouring blemish primarily of sapwood which can most often be removed by brushing the wood surface. However, the

discolouration on hardwoods is often more persistent. It differs from typical fungus stains in its comparatively shallow discolouration; mould fungi regularly penetrate deep into wood, but the discolouration occurs mostly at or near the surface (Zabel and Morrell, 1992).

Moulds tend to enter ruptured cells, vessels (hardwoods), and exposed rays, and spread from cell to cell via the pits. As they attack the pit membranes, these fungi make the wood more receptive to fluids. Treatments with moulds have been proposed as a method for improving the permeability of Douglas fir, the spruces, and other difficult-to-treat species (Schulz, 1956; Lindgren and Wright, 1954). ***Trichoderma*** colonisation, however, has also been shown to inhibit colonisation of pine pulpwood by decay and stain fungi during storage (Lindgren, 1952; Hulme and Shields, 1972 a). The principles behind this deterrent effect has inspired many workers including this author in exploiting this for possible biological protection of wood from decay fungi.

Moulds cause little damage to the structure of wood they inhabit, provided their action does not reach a more aggressive stage where it would be considered soft rot (Merrill and French, 1965; Nilsson, 1974). Moulding is heaviest on wood that has never been dried. Green lumber, timber, round wood, and veneer are most susceptible.

c) Staining fungi - Most staining fungi are in the Ascomycotina or Deuteromycotina. Many staining fungi are specific to a region or wood species. The staining fungi can be placed into two broad groups. Some of the fungi, and particularly those in the genera ***Ophiostoma*** and ***Ceratocystis***, are closely tied with the life cycles of bark beetles and other wood-inhabiting insects (Verrall, 1941; Dowding, 1969, 1970) since the spores are sticky and transmitted primarily by insects (vectors) and water splashing or aerosols. These fungi invade and damage wood primarily during log storage and the initial stages of lumber seasoning. The other group of stain fungi, such as ***Aureobasidium pullulans***, ***Alternaria alternata*** and ***Cladosporium*** spp. are general opportunists, whose dry spores are primarily disseminated in air. These fungi invade wood in a wide range of uses when conditions are conducive to fungal growth.

The hyphae of the staining fungi like the moulds also grow mainly in the ray parenchyma cells living on the proteinaceous content and readily available carbohydrates, but are also found in tracheids where they grow on the inner cell wall surface without any enzymic alteration of the cell wall structure.

Their growth within the wood is essentially limited to the sapwood. The discolouration on softwood products ranges from bluish black to steel gray. The predominance of a bluish discolouration and restriction to the sapwood have resulted in the major stains being termed blue stain or sapstain. On hardwoods these colours commonly are modified as brown shades. The discolouration may be faint or intense, depending on the conditions of its development and the species of wood (Zink and Fengel, 1988, 1989, and 1990). Another feature, observed on freshly exposed cross sections of softwood lumber, logs, and other round material, is a marked tendency of the stain to appear in radial streaks or wedged-shaped areas. This pattern results from the movement of discolouring fungi inward along the wood rays.

Protection of lumber from sapstain was largely accomplished by kiln drying or, temporarily, by surface treating with water solutions of conventional antistain fungicides like fluorides, boron and organic mercury compounds. This was followed by the use of sodium penta or tetra chlorophenate. But at present the organic mercury compounds are banned and the others have different restricted rules of usage in different countries due to the environmental hazards that may be associated with them. Compounds that show promise include oxine copper, triazoles, methyl thiobenzothiazole, methylene bisthiocyanate, and several quaternary ammonium compounds. However all these are quite expensive and not as effect as penta (Zabel and Morrell, 1992).

d) Soft rot fungi - Findlay and Savory (1950) reported a form of wood deterioration resembling brown rot decay by basidiomycetes but which differed from it in certain significant details. Later, observing that the deterioration could result in unusual softening of the wood, Savory (1954) applied the term "soft rot". Soft rot fungi,

comprise about 300 species including ***Chaetomium, Humicola*** and ***Phialophora*** species, of Ascomycetes and Deuteromycetes (Seehann ***et al.***, 1975, as cited in Schmidt and Kerner-Gang, 1986) which differ from brown rot and white rot basidiomycete fungi as they grow mainly within the woody cell wall. Wood colonisation is initiated through rays and vessels.

Soft-rot fungi preferentially attack cell wall carbohydrates in the S_2 layer of the secondary cell wall, forming longitudinal cavities (Type 1), or eroding the wood cell wall from the lumen surface in hardwoods or the S_2 in conifers (Type 2) (some bacteria are known to cause typical soft-rot cavities and related tunnel- or cavitation-type cavities in cell walls) (Corbett, 1965; Leise, 1970). In the type 1 soft rot of softwoods, small perforation hyphae penetrate from the tracheidal lumina through the S_3 layer and grow after 'T-branching' longitudinally within the S_2 layer. Within the S_2 layer, fungi often produce hexagonal-shaped cavities, microscopically visible in polarised light, which follow the orientation of the microfibrils and are arranged like pearl strings. In cross sections, cavities are oval-shaped and develop with progressive decay into large wall openings. Due to intense lignification, the S_3 layer and the compound middle lamella remain unaffected by soft rot decay (Figure 1.2) (Nilsson ***et al.***, 1989).

In hardwoods, type 2 soft rotters after eroding the cell wall from the lumen via S_3 layer, they burrow towards the compound middle lamella, accompanied by hyphal slime covers, which is both characteristic of white rot fungi (Schmidt and Kerner-Gang, 1986). Timber in service attacked by soft rot retains macroscopically its shape and become spongy in texture with decay proceeding slowly from the outer wood parts towards the center.

There are some inconsistencies and neglected areas in this grouping of the four major decay types. For example, some basidiomycetes form small cavities in the cell wall. These resemble Type 1 soft-rot attack, and the cell wall erosion of the Type 2 soft-rotters in hardwoods resembles an early stage of white rot. Recent studies of decay

caused by several of the Xylariaceae (Ascomycotina) have shown that wood damage by these fungi is similar to that produced by the simultaneous white rots (discussed later).

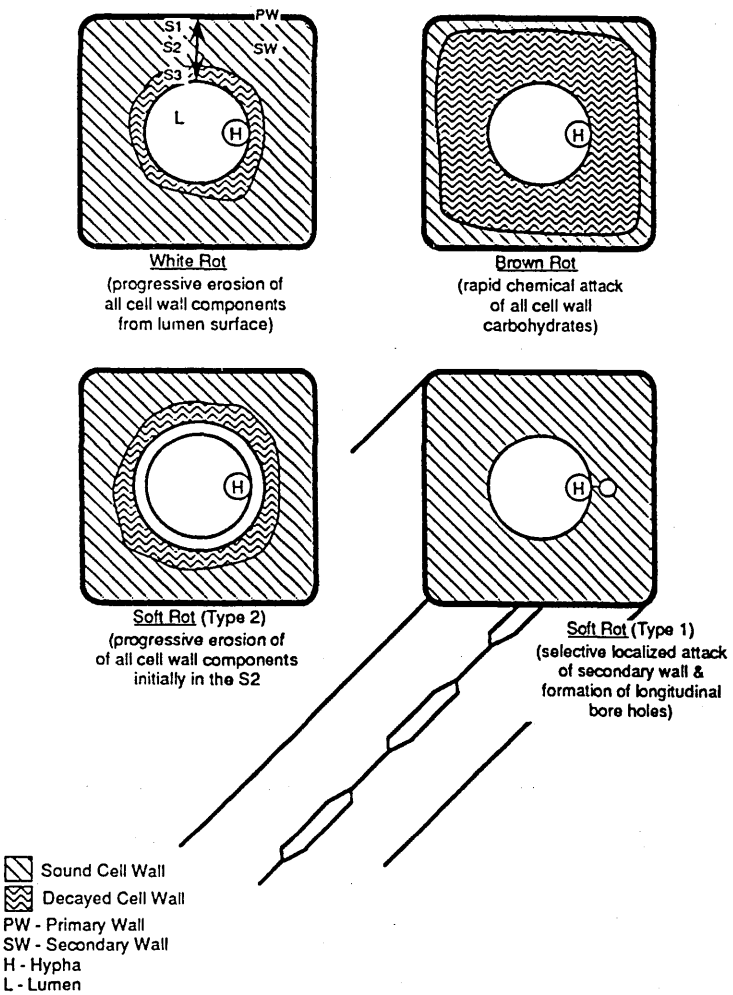


Figure 1.2 - Diagrams showing the various modes of cell-wall destruction for white rots, brown rots, and the two types of soft rots (modified figure from Zabel and Morrell, 1992).

under the white rot fungi) but also contains typical soft-rot cavities (Kistler and Merrill, 1968; Nilsson ***et al.***, 1989).

Within the woody cell wall, soft rot fungi consume cellulose and hemicellulose. Lignin is attacked only to a small extent. The greater tendency of hardwoods to decay as compared to softwoods and the preference for latewood tracheids of conifers result from differences in amount and type of lignin in these timber groups and from the lignin distribution within the woody tissue. The composition of the hemicellulose portion also influences soft rot fungal activity (Montgomery, 1982).

Soft rot fungi decay wood under stressful micro-environmental regimes (i.e., permanently wet wood, such as cooling towers, harbour posts and ships). Wood constantly in ground contact, such as transmission poles, railroad ties, fence posts, is also affected as in wood in the center of chip piles. Fungal requirements for wood moisture range widely from fiber saturation point (with the ability to survive under dry conditions) up to growth in nearly water saturated wood. The varied temperature range of soft rot fungi includes thermophilic species and enables them to destroy the warm timber in sunny windows (Schmidt and Kerner-Gang, 1986). Soft rot fungi are also capable of growing at extreme pH ranges for example, ***Chaetomium globosum*** start growth at pH-values between 3 and 11. Soft rot decay is however limited by the soluble nitrogen content that is present in wood (Savory, 1954). King ***et al.***, 1974 have shown that during drying of wood, soluble nitrogenous materials and carbohydrates migrate with water to wood surfaces where they are deposited after evaporation. However, since much of the nitrogen is in the form of wood cell wall protein, it is not available to microfungi until breakdown has occured. Microfungi are therefore dependent on soluble nitrogen before they can decay wood, hence the form and distribution of nitrogen in wood probably exercise a controlling influence on their development. This was confirmed by several workers, Garrett (1963), Findlay (1966) and Levy (1968) who observed most active soft rot decay in timber that were in contact with soil or with water containing nutrients in solution.

Soft rot fungi degrade also the heartwood of timber species which possess a high natural resistance to other fungi such as bongossi or teak. Furthermore, these fungi are highly tolerant of chromated fluorine salts which are used for the preservation of weathered wood to brown and white rot fungi. Serious problems occur with hardwoods. In Australia, for instance, hundreds of thousands of eucalyptus transmission poles, treated with chromated copper-arsenic, showed premature failure by soft rot (Schmidt and Kerner-Gang, 1986).

e) Basidiomycetes - The members of this group are regarded as the major wood degrading fungi. Two major types of decay are recognised based on action within the wood and on reaction of the causal fungus to tests for extracellular oxidase (Nobles, 1965). The major subdivision into white and brown rots was made in 1874 by Hartig, based on the colour of the residual woods and the assumptions that whitish material was cellulose (white rot) and the brownish material, lignin (brown rot). Subsequent research established that white-rot fungi actually utilised all cell-wall constituents (cellulose, hemicellulose, and lignin) (Campbell, 1932; Scheffer, 1936), but the early dogma persisted for many years. The grouping of decay into white- and brown-rot categories remains a major subdivision, reflecting very different chemical processes in the decay of wood.

Like all other fungi the wood-inhabiting fungi have certain growth requirements for survival. These include free water, i.e., water which is present on the surface of cell lumina; atmospheric oxygen at relatively low levels for most fungi and very low levels or chemical oxygen only for some microaerobic and facultative anaerobic fungi; a favourable temperature range, optima for most the basidiomycetes range from 20 - 36 C ; a digestible substrate (wood) that provides energy and metabolites for synthesis via metabolism; a favourable pH range, the optima for basidiomycetes range from 3 to 6 ; and other chemical growth factors like nitrogen compounds, vitamins, and essential elements that are often included with the substrate.

Brown rot fungi

Brown rotted wood is so described since after utilisation of the homocellulose, the fungus leaves only the brown, brittle and friable residue of lignin. These fungi invade the wood through the rays and spread through both the pits and by transverse penetration with microhyphae throughout the tracheid walls. In contrast to soft rot fungi brown rot fungi grow mainly within the cell lumina in close contact with the surface of the tertiary wall.

Within wood, cellulose depolymerisation by brown rot fungi requires a pre-cellulolytic phase (Bailey ***et al.***, 1968) which makes cellulose fibers accessible to cellulases and this is thought to occur through the action of Fe^{2+}/H_2O_2 (Koenigs, 1974). Which is increased if the hemicelluloses are removed. The predecomposing agents and the enzymes penetrate the more resistant tertiary wall and diffuse into the S_2 layer where they remove the carbohydrates totally and leave the lignin as a skeleton (Figure 2). A more detailed review of the enzymic degradation by the brown rot fungi is given in chapter 4.

Wood strength properties may be reduced through fungal carbohydrate depolymerisation by about 50% during early decay when mass losses account for only a few percent. As parasites some brown rot fungi may attack living trees through the roots eg. ***Phaeolus (Polyporus) schweinitzii*** (Scheffer ***et al.***, 1941) and ***Sparassis crispa (radiata)*** (Siepmann, 1976), or via stem wounds like ***Laetiporus sulphureus***. Various other brown rot fungi like ***Gloeophyllum***spp., (Silverborg, 1953; Cartwright and Findlay, 1958; Duncan and Lombard, 1965; Walters, 1973) and ***Paxillus panuoides*** (Coggins, 1980) decay stored timber. Degradation of lumber in service outside also occurs e.g., by ***P.panuoides*** in mining timber. The fungus ***Neolentinus lepideus*** destroys the heartwood of creosote-treated poles and ties by reaching it through crevices which occur after impregnation of improperly seasoned wood (Cartwright and Findlay, 1958; Bruce and King, 1983). The fungus tolerates dry and warm conditions in latent state and has a high resistance to creosote. ***Daedalea quercina*** decays oak heartwood which is generally resistant to most fungal degradation (Cartwright and Findlay, 1958;

Coggins, 1980). ***Gloeophyllum abietnum*** and ***G.sepiarium*** are important brown-rotters of softwood joineries where they can survive periods of dryness and heating up by sunshine (Lea and Bravery, 1986).

Major brown rot fungi in buildings (Coggins, 1980) are ***Coniophora puteana*** which produce fine blackish strands and is found in fresh wood of buildings, ***Fibroporia vaillantii*** and other ***Poria*** spp. (Eslyn and Lombard, 1983) form white flexible strands. The 'true dry rot fungus' ***Serpula lacrymans*** (the name given is deceiving as they are unable to grow in wood with less than 20 % moisture content) are found in older houses in cool temperate regions, usually in basement and ground-floor timber and form thick grayish strands (Theden, 1952, Coggins, 1980). The last mentioned fungus causes most damage as it is able to grow by its strands through non-woody materials such as mortar layers between stones and to transport water from moist wood to dry wood (below fiber saturation point) which facilitates its rapid spread throughout buildings in temperate climates (Walchli, 1980).

White rot fungi

Degradation of the cellulose, hemicellulose and lignin by the white rot basidiomycetes results in bleaching of the wood. Depending on the fungal species and stage of decay, the degradation of carbohydrates and lignin can occur either simultaneously (***Trametes versicolor***) or selectively with faster lignin degradation in the early stages of decay (***Heterobasidion annosum***).

Cell wall deterioration can occur through production of bore-holes which develop with progressing decay into larger wall openings. Mainly however hyphal growth on the surface of the tertiary wall in the lumina result by enzymatic activity in the production of erosion troughs. Enzyme action is restricted to the vicinity of the hyphae, which are embedded in a slime layer and produce lysis zones (Leise, 1970). The S_2 layer is more rapidly degraded than the tertiary wall and the compound middle lamella (Figure 2). The macrostructure of the remaining wood may look a white pocket hole. The actual enzymic mechanism of white rot decay is reviewed in chapter 4.

White rot occurs predominantly though not exclusively in hardwoods where fungi such as the ***Heterobasidion fomentarius*** are involved in the decay of old and weak trees. Important forest pathogens are the honey fungus ***Armillaria mellea*** (Kile, 1981; Thompson and Boddy, 1983) which parasitically attacks a wide range of hardwoods and softwoods and forms special strands (rhizomorphs), and ***Heterobasidion annosum*** (Chase and Ullrich, 1983; Stenlid, 1985) (butt rot of conifers). Various white rot fungi have a narrow host range or are specialised on heartwood, e.g., ***Polyporus squamosus*** are found causing top rot mainly on Elm, Sycamore and Walnut (Rayner and Boddy, 1986), while ***Stereum*** species (Boddy and Rayner, 1982) are slow white rotters in stored timber such as Oak. Pulp wood chip piles may also suffer from white rot (Bjorkman and Haeger, 1963). ***Schizophyllum commune*** (Rayner and Hedges, 1982) and ***Trametes versicolor*** (Cartwright and Findlay, 1958) are ubiquitous fungi, the latter being one of the most vigorous wood destroying fungi causing up to 95% weight loss in the wood blocks used under laboratory conditions. As construction wood is mainly made from softwoods, white rot fungi occur rarely within buildings (Schmidt and Kerner-Gang, 1986).

f) Secondary moulds - These fungi do not appear to alter the structure of wood but do possess an active cellulase system. Their position in the succession pattern of organisms in wood as final colonisers seems to be associated with the appearance and eventual dominance of the decay fungi, particularly the basidiomycetes. The role of these secondary colonizers, predominantly ***Trichoderma viride*** and ***Gliocladium roseum***, is probably one of utilising the excess cellulose sugars derived from the breakdown of the wood, by the decay fungi. This cellulose food source may be a true nutritional excess, or become available as a result of competition between the two groups of organisms for the partially decayed substrate (Levy, 1982).

1.6.3 Wood preservation

As the value of the raw material, wood, is constantly rising and wage costs for repairing and renewing attacked wood are increasing, it is therefore economically important to prolong the service life of wood.

Wood-destroying fungi especially soft rots and basidiomycetes present the greatest hazard to the economic use of wood. The importance of controlling them has been recognised since man's earliest times. By the middle of the eighteenth century it was known that copper sulphate stopped decay (Boulton, 1744), and 50 years later Chapman (1817), illustrated the interest in the use of chemical control methods when he complained that "almost every chemical principle or compound of any plausibility has been suggested in the course of the last five years and submitted either to the admiralty or navyboards; but the multiplicity and contradiction of opinions formed nearly an inextricable labyrinth". Thus, over 100 years ago, before Hartig had discovered that decay was caused by fungi (1874), it was known that decay could be controlled either by keeping wood dry or by treating it with certain chemicals (Refs as cited in Schmidt and Kerner-Gag, 1986).

The relative values of the end products generally determine the nature and cost viability of the feasible control treatments. In todays modern society four main groups of protecting methods are common:

1) 'Natural methods' are based on naturally decay-resistant wood. Decay resistance varies widely among species e.g., species such as cypress and cedars are very durable in comparison to beech and maple. Decay resistance is highest near the sapwood-heartwood interface and decreases toward the pith for many durable species. The nature and amount of toxic extractives in the heartwood appears to be the major factor affecting decay resistance. The primary toxic heartwood extractives are the polyphenols, terpenoids, tropolones, and tannins. The use of durable woods as replacements for preservative-treated materials may increase in some environmentally sensitive areas.

However, such durable timbers are becoming more expensive due to the increasing demands.

2) Protection of green timber during storage can be achieved by either rapid utilisation, arranging the major storage period to begin during a cold season; storage under water; and water sprinkling or spraying. The actual controls practised vary greatly with species, periods of storage, end product use, region, and size and sophistication of the industry. For low-cost items with short-term storage, practices are followed to keep the units wet; for long-term storage, the wood is peeled so it can dry out as rapidly as possible. For high-cost items water immersion is necessary. Wood that has survived the storage period and is used in construction can also be saved from decay by following special construction practices that avoid conditions such as dampness (Zabel and Morrell, 1992).

The methods of protection discussed above are purely physical preventitive practices, the most common method for protection of high-cost and long-term usage or storage of timber is chemical treatment. Chemical preservation is necessary if the above methods are inadequate. For example, the expected service life of beechwood railroad ties is 45 years or even more when properly treated, but only 2 years when untreated (Schmidt and Kerner-Gag, 1986).

Specific chemicals and treatments are recommended according to the 5 hazard categories of wood:- above ground covered (dry), above ground covered (risk of wetting), above ground not covered, in contact with ground or fresh water and in salt water. Such hazard groups are based on the European wood decay hazard categorisation as found in EN 335 specifications.

Chemical preservatives for wood can belong to 3 categories:

1) Water-borne preservatives - These are mostly inorganic salts, such as fluorides or borates, which are used only within buildings as they are leached by water. Other salt

mixtures contain chromium which fixes the components to the woody tissue. Chromated fluorides (CF) or fluoroborates (CFB) are used for outside wood that are not in contact with the ground. Wood in constant ground contact (e.g.,poles) or in flowing water (e.g., cooling towers) need to be preserved with a more permanently fixed preservative such as chromated copper salts (CC) to be protected against soft rot fungi and additionally arsenic (CCA), boron (CCB), or fluoride (CCF) against the various basidiomycetes. The CC-wood preservatives are also strong inhibitors of bacteria. All these chemicals are in use in various parts of Europe however, CCA is under restricted use in the USA (Zabel and Morrell, 1992).

2) Oilborne Preservatives - Different distillates of coal tar oil (creosote) due to their volatile character are only used for timber outside, mainly in a heavy variant for railroad ties and in a lighter consistency for transmission poles or posts. Another oil-borne fungicide Pentachlorophenol(PCP) was used in the form of sodium-pentachlorophenol for the protection of green timber during transport up to processing in some countries, but is still in use in the UK. It has been largely replaced due to possible health risks associated with its use. Copper Napthenate, a copper complex of naphthenic acids derived from the oil-refining process, has recently been promoted as a PCP replacement. It has been reported to be 20 times less toxic to humans than penta (Zabel and Morrell, 1992).

3) Organic solvent based wood preservatives - which contain organic biocides , such as tributyltin oxide, Xyligenes as fungicides, and Lindane or pyrethroides as insecticides, have been employed for inside and outside use for out of ground contact situations. Internal use of such biocides however may be restricted by national regulations which may vary from country to country.

Tributyltin oxide (TBTO) is used extensively in Europe to protect window and door frames and in many marine paints. In the United States, TBTO has been used primarily for treating millwork, particularly where rapid removal of the solvent treatment is desired. It has not performed well in ground contact, where fungi have been shown to

degrade this chemical. Recent reports about the damage to shellfish from TBTO leaching from antifouling paints may lead to further restrictions on the use of this chemical in most countries.

Copper-8-Quinolinolate also known as Cu-8 or oxine copper, has the lowest mammalian toxicity of any wood preservative currently used for wood treatment (U.S. Food and Drug Administration). It is the only preservative approved for direct food contact. However, its high cost and questions about its performance in direct ground contact have largely limited its use.

In addition to the above categories for special products and uses additional preparations exist such as fumigants or oil-salt mixtures (Zabel and Morrell, 1992).

Application of preservatives depends on the suitability of the timber species which varies from treatable types such as pine sapwood to refractory species such as spruce and also the required time period of protection. The various methods may be grouped into 3 categories:

1) Short-term treatments, involving dipping or spraying of fungicides onto lumber or log surfaces, are available to protect logs and lumber from sapstains and early decay during storage or seasoning.

2) Long-term treatments are necessary for wood used when conditions conducive to decay prevail. These treatments are often subdivided into nonpressure and pressure treatments.

The nonpressure treatments include dipping, brushing, spraying, and soaking the wood in a preservative. This treatment type provides only a thin shell of treated wood and provides useful protection to wood exposed above ground and subject to intermittent wetting. It is of minimal value to wood in ground contact and generally not

recommended. Pressure treatments are necessary to protect wood exposed to decay conditions in high value structures.

Pressure treatments use combinations of pressure, vacuum, and elevated temperatures to force the preservative solutions deep into the wood. Long-term protection requires that the penetration exceed the depth of subsequent cracks that are potential infection sites. There are many treating schedules which involve use of pressure. These use pressure treatments of up to 150 - 200 pounds per square inch (psi) (1050-1408 kPa) which may be applied between certain intervals of time and follow special schedules according to type of preservative and timber species. They yield deep penetration and high retention of the preservative. Double vacuum treatment for semi-finished wood products uses alternating vacuum and low air pressure (Schmidt and Kerner-Gag, 1986; Zabel and Morrell, 1992).

3) Remedial treatments such as ground line pastes, or fumigants are useful for arresting decay that occurs in treated wood in service.

Wood preservatives are tested and compared for their effectiveness by petri-plate and soil-block tests (European standard tests that allow assessment of toxicity of individual preservatives against wood decay basidiomycetes (EN 113) and soft rot fungi (CEN/TC 38 N682) and other equivalent national and international standard tests), and the exposure of treated stakes in decay cellars, field-stake tests,or wood-assembly tests. However, after many years of preservative use tolerance or resistance to wood preservatives has developed among some of the common decay fungi e.g., resistance of the brown rot fungi ***Postia placenta*** to copper naphthenate (Zabel, 1954) and white rot fungi ***Irpex lacteus*** to creosote (Cowling, 1957). The resistant strains of decay fungi are generally used as test fungi in the evaluation of new wood preservatives. Information on the tolerant decay fungi associated with a specific wood use is valuable information with regard to the selection of the preservative and its retention level. Major research is needed in the development of effective, economical, and environmentally acceptable wood preservatives. The exploration of biological control provides a possibility for the

future protection of the enormous amounts of wood that is used under conditions conducive to decay.

1.7 Biological control in wood

Preservative treatment systems, currently in use for decay in wooden structures, involve the application of toxic chemical compounds into wood. However, as a result of increasing pressure from environmentalists, who are concerned about the harmful effects of such chemical biocides, wood preservative companies have been forced to seek alternative environmentally acceptable treatment technologies. Biological control may provide one such alternative, either in conjunction with less toxic chemical treatments or as a complete treatment. Although to date there are few examples of commercially successful bioprotection systems for wood products, the pressure to reduce chemical application suggests the need for continual evaluation of these systems. This mirrors a similar trend in agriculture where removal of registration of various soil fumigants has left a vacuum for efficient control and lead to the development of appropriate biological control systems. Biological control research in agriculture has contributed valuable information towards the development of similar control systems in wood, however some vital differences exist between the control of plant pathogens and wood decay fungi as highlighted by Bruce, in Palfreyman and Bruce (1993). Some of these differences are outlined below :

i) Period of protection

Control of plant diseases may require in some cases to be effective over just the one growing season. In wood, prevention of decay by bioprotectants may have to remain effective for anything up to 40 years depending on the expected service life of wooden structures.

ii) Efficacy of the system

In agriculture it may be reasonable to accept a 95% yield using biocontrol in comparison to a 100% yield with chemical control, especially if other constraints eg. legislation make the biological control an attractive alternative. The same is not applicable in wood

however, as the strength and stability of wooden structures are often compromised even with minimal wood degradation.

iii) Availability of nutrients

Though soil may not be classed as an ecosystem with high nutrients, its resources are replenished by regular supplies from the plant residues and exudates, wood however lacks this renewable supply of nutrients. This has major implications on the development and use of biocontrol since extended growth may be limited by a lack of nutrients.

iv) Delivery systems

In agriculture delivery systems for biocontrol agents are similar to those used for chemical pesticides, but the same transfer of technology cannot be expected for wood preservatives as they are generally impregnated at high temperatures which would be totally unsuitable to deliver living organisms. Therefore new avenues need to be explored for the development of appropriate delivery systems in wood.

v) Target specificity

Once wood is felled a range of organisms can colonize and degrade it. The same situation also exists in agriculture but the plants natural defense mechanisms reduce the range of pathogens which any subsequently applied biocontrol agent would have to control. In wood the biocontrol agent may potentially have to control more than one pathogen and indeed in some instances may be expected to control a wide range of decay agents.

The choice of any biocontrol agent is very much limited by the organisms ability to establish itself in the environment of its target. There are a number of biocontrol agents that are currently at or near to commercial use in the agricultural industry as stated earlier, however, few if any have been commercialized for preventing wood decay.

One reason for this slow rate of progress may relate to concerns about the potential efficacy of the process and the practical difficulty and expense of setting up the necessary field trials to test biocontrol products. While many laboratory investigations have shown antagonism between potential control agents and wood decay organisms only a limited number of field trials have been undertaken. Since wood is a relatively low nutrient environment a high percentage of control agents successful in autecological studies might not repeat the successful control on transfer to the field. In addition all too often the exact mechanism of the antagonism between the control and decay agents has not been identified making appraisal of field performance even more difficult.

Earlier literature cites several instances where researchers noted interactions in which one fungus apparently limited attack by another in wood, but direct attempts to exploit organisms for this purpose as biological control, began only in the early 60's. The use of fungi to protect wood from decay was advanced by a number of researchers (Ricard and Bollen, 1967; Nelson, 1969; Kallio, 1971; Hulme and Shields, 1972 b) based on findings in control of agricultural pathogens (Baker and Cook, 1974).

In a biological control project, a control agent is introduced into the wood either through a freshly cut stump, or by inoculation into a hole drilled in the wood. In practice, control of established pathogens is extremely difficult, and most strategies seek to prevent colonisation or protect the host from attack. Thus, biological protection is a more appropriate term for this strategy (Freitag ***et al.***, 1991). The control agent then colonises the wood to prevent any subsequent fungal invasion by the pathogens The use of bioprotection is based on two critical points; the fungus must be capable of completely colonising the substrate without damaging the wood, and the protection must be long-term. These two requirements have severely limited the list of potential bioprotectants (Preston ***et al.***, 1982).

The first attempts to employ bioprotection in wood were directed at preventing fungal colonization of freshly cut stumps by ***Heterobasidion annosum*** with application of ***Peniophora gigantea*** (Rishbeth, 1963). Subsequently Ricard ***et al.***, (1969), Hulme and

Shields (1972 b) and Graham (1973) all examined the use of biocontrol to protect felled timbers and finished wood products. Ricard and Bollen (1967) used ***Scytalidium*** sp. to inhibit ***Antrodia carbonica*** in Douglas fir poles. They reported that an antibiotic produced by ***Scytalidium*** was responsible for control of the wood decay fungi. Although effectiveness of this agent was debated, the findings stimulated additional research.

Several workers found that ***Trichoderma*** sp. inhibited the growth of wood-decaying fungi (Glaser ***et al.***, 1959; Lindgren and Harvey, 1952; Sheilds and Atwell, 1963). In one series of experiments performed by Sheilds (1968), freshly cut ends of birch logs, 4 feet in length, were sprayed with a spore suspension of ***Trichoderma viride*** and two weeks later sprayed with a spore and mycelial suspension of ***Polyporus adustus***. The logs were then stored from May until late November and, at the end of this period, fungal isolations were attempted. ***Trichoderma viride*** almost competely prevented colonisation by the wood-destroying fungus. Bolts sprayed only with ***Polyporus adustus*** yielded many isolates of that fungus, and control bolts sprayed with water yielded a variety of wood-destroying and wood staining fungi. Earlier experiments by the same authors (Shields and Atwell, 1963) gave less successful control, and in some bolts unidentified basidiomycetes had caused attack in ***Trichoderma***-sprayed bolts. It was suggested that ***Trichoderma*** inhibits the attack of wood-destroying fungi by removing some structural carbohydrates from the wood which are necessary for rapid colonisation and initiation of decay by wood-destroying fungi and that the mechanism did not appear to involve antibiotic production (Hulme and Shields, 1970).

Much of the research work since then has concentrated on the use of ***Trichoderma*** spp. as possible control agents for wood decay fungi (Bettuci ***et al.***, 1988; Bruce, 1983; Bruce ***et al.,*** 1990; Bruce and King, 1986 a,b ; Highley and Ricard, 1988; Morrell and Sexton, 1988; Morris, 1983; Murmanis ***et al,*** 1988 b; Nelson and Theis, 1985; Seifert ***et al.***, 1988). Reviews articles have also been published on different aspects of ***Trichoderma*** by Eveleigh (1984), Papavizas (1985) and Chet (1987). ***Trichoderma*** has received much attention because of the encouraging results with this genus in earlier studies (Hulme and Shields, 1970, 1972 b; Ricard ***et al.,*** 1969) and the fact that

Trichoderma spp. have been widely studied as potential control agents for a wide range of plant pathogens in agricultural systems (Papavizas, 1985; Tronsmo, 1986). Other micro-organisms have also been studied for application as biological control agents to protect wood decay including bacteria (Benko, 1988; Bernier *et al.*, 1986; Preston *et al.*, 1982) and other microfungi (Highley, 1989; Kellock and Dix, 1984; Morris and Dickinson, 1981; Seifert *et al.*, 1988).

Although successions and interactions of *Trichoderma* and many pathogens have been studied extensively (Shigo, 1967; Eslyn, 1970, 1986; Tolle, 1971; Greaves, 1972; Smith, 1973; Kaarik, 1975; Rayner and Todd, 1977; Zabel *et al.*, 1980, 1985; Gramss, 1987; Przybylowicz *et al.*, 1987; Chapela *et al.*, 1988), few reliable bioprotection or biocontrol systems have been generated from this research. This is also likely to be due to the difficulties that are associated with production of bioprotectants against wood decay in comparison to plant pathogens as discussed in section 1.7.

The use of biological agents to control decay of creosoted distribution poles using *Trichoderma* and *Scytalidium* species has been reported by a number of authors (Ricard, *et al.*, 1969; Ricard, 1976; Bruce, 1983; Morris, 1983; Bruce and King, 1986 a, b; Bruce *et al.*, 1990). These field studies, have shown variable, and limited success compared with favourable laboratory observations of antagonism between *Trichoderma*, *Scytalidium* and some wood decay organisms common in creosoted wood poles. Highley and Ricard (1988) showed that wood blocks treated with *Trichoderma* species could be protected from attack by selected brown rot fungi.

Bruce and King (1983) found that wood blocks could be protected from *Neolentinus lepideus* by *Trichoderma* spp. even after killing the *Trichoderma* and leaching the wood. Morris *et al.*, (1986) confirmed this residual protection against *N.lepideus* but found that the decay resistance was lost after extended leaching. Morrell and Sexton (1988) however, found only limited success by *Trichoderma* in arresting the development of decay fungi in wood blocks. Bruce, King and Highley (1991) showed that wood blocks removed from a distribution pole previously treated with a biological

control product (Binab FYT pellets - seven years after treatment) when exposed to soil block tests with selected basidiomycetes could resist decay. The blocks were removed from regions of poles where ***Trichoderma*** colonisation had been confirmed by extensive sampling and computer mapping of microbial inhabitants. Any decay prevention was lost however when the wood was steam sterilised prior to exposure to the basidiomycetes.

A Swedish bioprotection formulation, Binab T was developed by Dr. J. Ricard which has been marketed in Europe for protecting Scots pine and later commercialized for use in the United States. The pellets of Binab contain mixtures of the mycelium and spores of naturally occurring species of ***Trichoderma polysporum*** and ***T.viride***. The potential use of Binab T pellets include suppression of wood infesting organisms such as ***Poria carbonica*** and ***Neolentinus lepideus*** for utility poles, fence posts, and wood playground equipment. Many researchers have worked with such pellets and there is considerable debate concerning the long-term effectiveness of this formulation (Morris ***et al.***, 1984; Morris and Dickinson, 1981; Bruce and King, 1983).

Recent tests on Southern pine and Douglas fir suggest that this bioprotectant cannot completely control the numerous decay fungi associated with these species (Morrell and Sexton, 1990). In addition, the agent was unable to completely eliminate decay fungi already established in the wood, nor did it perform well against white-rot fungi. Brown-rot fungi are an important component of many decay systems, but recent studies have shown that white-rot fungi are far more common in coniferous woods than previously thought (Zabel ***et al.***, 1982; Graham and Corden, 1980; Eslyn, 1970). The influence of resident wood organism on the colonisation of an introduced biocontrol agent was evident in the extended field studies done by Bruce and King (1986 a and b). They inoculated 40 recently creosoted transmission poles with either ***Trichoderma*** (in the form of Binab FYT pellets) alone, ***N. lepideus*** alone, or ***Trichoderma*** followed by ***N. lepideus***, and tried to reisolate these after 0-18 months; it was observed that the spread of ***Trichoderma***, and to a lesser extent ***N. lepideus***, was influenced by fungi already

resident in the poles, e.g. ***Cladosporium resinae***, leaving areas uncolonised by the biological control agent which would be open to attack by ***N. lepideus***.

As discussed earlier in section 1.7 agriculture can withstand incomplete protection (as measured by yield loss in disease control), however the presence of small amounts of decay that can subsequently enlarge to destroy additional wood cannot be tolerated in a large wood structures. As a result bioprotection does not appear to be feasible without the use of supplemental treatments that alter the ecology of the wood to favour growth or activity of the bioprotectant. For example, chemical pretreatments to eliminate any competing fungi may provide an edge to the bioprotectant, which is applied some period of time after chemical treatment (remedial treatment).

1.8 Aims and Objectives

The major aims of this research programme were to identify the antagonistic mechanisms that ***Trichoderma*** spp. employ against wood decay fungi and to determine which are likely to be most important during biocontrol of wood decay fungi ***in vivo*** i.e., in wood.

Though much work has been undertaken to engineer better biocontrol agents for wood, the rate of progress has been slow and this has increased the concerns of end users over the potential efficacy of biocontrol processes. Although many laboratory investigations have shown antagonism between potential control agents and wood decay organisms, only a limited number of field trials have been undertaken. The uncertainities and differences between the results of field and laboratory experiments may be due to a number of factors and more information is required to allow full exploitation of this developing technology. This is particularly important with regard to the antagonistic mechanisms of the biocontrol agents.

The major objective of this study was to identify the principle antagonistic mechanism or mechanisms involved in the control of selected wood decay organisms by

Trichoderma spp. Although various modes of antagonism employed by ***Trichoderma*** spp. against wood decay basidiomycetes including competition for non-structural carbohydrates, production of soluble metabolites, volatile antibiotics and lytic enzymes have been identified by many workers (See sections 3.1.1, 3.2.1, and 3.3.1), experimentation has largely involved studies on agar media that shows little correlation to wood with respect to nutritional consistency. Studies of antagonistic mechanisms carried out on such artificial media are therefore likely to be unrepresentative of results seen during interactions in wood.

A primary objective of the study was therefore to devise an appropriate media for all the experimental studies and to determine whether screening or study of the antagonistic mechanisms of ***Trichoderma*** isolates is influenced by the use of media that is not representative of the nutritional state of the ***in vivo*** substrate i.e. wood. As an extension of the studies on media composition, the project was designed to determine whether any statistical correlation exists between the degree of inhibition by different antagonistic mechanisms on artificial media, and the protective effect attained when different ***Trichoderma*** isolates were tested against selected basidiomycetes on wood.

Though extensive work has been done on the role of mycoparasitism between ***Trichoderma*** and plant pathogenic fungi, little has been published with regard to its role against wood decay fungi. Due to the importance given to this particular mechanism against agricultural pathogens, one of the objectives of the project was to study the production of the lytic enzymes involved in mycoparasitic interactions against wood decay fungi. Again the majority of the work done in this area has involved the use of inappropriate media that do not represent the nutritional status ***in vivo***. In order to entirely understand the importance of the role of lytic enzymes, assaying in an appropriate media that is more representative of the nutritional consistency of wood was therefore essential. An additional objective was to examine factors such as target cell wall type and glucose concentration, which may influence the induction and repression of lytic enzymes produced as part of any mycoparasitic reaction.

Siderophores (iron chelating compounds) are known to play an important role in the biocontrol of many plant pathogenic fungi and bacteria. To date however, no work has been published regarding iron competition in wood as a possible antagonistic mechanism against wood decay fungi. Iron plays an important role in the biodegradation of wood both as a component of extracellular heme enzymes in white rot fungi and may also possibly have a role in non-enzymatic decay by brown rot fungi. Due to the very low concentration of iron in wood, siderophore competition for iron between ***Trichoderma*** and the decay fungi may therefore be a very significant biocontrol mechanism. A further objective of the project was therefore, to determine whether the production of siderophores by ***Trichoderma*** isolates could be involved in the biocontrol of wood decay fungi.

This thesis is designed to provide fundamental information on the vital antagonistic mechanisms that are involved during the interaction phase between antagonist and wood decay fungi. Such information on species and strains of ***Trichoderma***, will enable selection of bioprotectants or biocontrol agents with better performance; which can be improved by genetic manipulation and provide a more appropriate basis on which to screen new isolates. This is a newly developing field that has attracted many researchers because it provides an environmentally safer alternative to chemical preservatives. Knowledge developed from studies such as this one will hopefully promote the acceptability of the technology through the development of better biocontrol agents.

Chapter 2

Interaction Studies

Chapter 2

Preliminary Work - Interaction Studies

2.1 Introduction

Screening of ***Trichoderma*** species as potential biocontrol agents for wood decay fungi has largely employed work on artificial media using agar plates though some researchers have developed wood wafer screening systems for this purpose (Frietag and Morrell, 1990; Dawson-Andoh and Morrell, 1991). Agar systems provide a fast, simple, cost effective method of screening a large number of isolates and produce quantifiable data which will allow suitable statistical analysis to be carried out. The major disadvantage however is that they do not accurately reflect the nutritional status or physical characteristics of wood. Wood is a substrate with a particularly high C:N ratio (350:1 to 1250:1) whereas a common medium for the growth of fungi such as malt extract medium has a much lower C:N ratio (50:1) (King, 1981).

A low nutrient media (based on a nutrient solution by Huttermann and Volger, 1973) with a C:N ratio similar to that found in wood (approximate C:N ratio 410:1) was therefore used for the screening work along with malt extract medium. Screening involved study of the outcome of interactions between the selected ***Trichoderma*** isolates and the two chosen basidiomycetes, the brown rot fungus ***Neolentinus lepideus*** and the white rot fungus, ***Trametes versicolor*** (both are considered to be major decayers of timber in ground line contact, the former of softwoods and the latter of hardwood, however it has become increasingly clear that white rotters are also capable of producing considerable damage to softwoods, Zabel ***et al.***, 1982; Graham and Corden, 1980; Eslyn, 1970). The marked differences observed in the two media types with respect to the outcome of the interactions is reported and also the basis of selection of ***Trichoderma*** isolates for further study of mechanisms of antagonism.

2.2 Materials and Methods

2.2.1 Organisms and Culture Conditions

The cultures used in the following studies were provided by the following sources : Scottish Institute for Wood Technology, Dundee Institute of Technology, Dundee; IMI (International Mycological Institute, Kew, U.K.), MAD and HHB (Madison Forest Products Laboratory, Wisconsin, U.S.A.), FPRL (Department of the Environment Building Research Establishment, Garston, U.K.), and E.E.Nelson, USDA Forest Serv., Pacific Northwest Res. Sta., Corvallis, Oregon.

Trichoderma Isolates (Optimum temperature for growth : 25 °C)

T.aureoviride (Rifai) IMI91968 *

T.harzianum (Rifai) IMI206040

T.polysporium (Rifai) IMI206039

T.viride (Persoon ex S.F.Gray) IMI24039

T.viride (Persoon ex S.F.Gray) IMI49791 *

Unidentified ***Trichoderma*** CCA sample (Isolated from a CCA treated pole)

Unidentified ***Trichoderma*** FYT sample (Isolated from a BINAB pellet)

T.viride (Persoon ex S.F.Gray) IMI335517

T.harzianum (Rifai) IMI335518

T.pseudokoningii (Rifai) Isolate 33

T.pseudokoningii (Rifai) Isolate 22

T.harzianum (Rifai) Isolate 25 *

T.pseudokoningii (Rifai) Isolate 33

T.pseudokoningii (Rifai) Isolate 51 *

T.pseudokoningii (Rifai) Isolate 55 *

T.pseudokoningii (Rifai) Isolate 64 *

Unidentified ***Trichoderma*** Isolate 140 *

T.citrinoviride (Rifai) IMI335519

Trichoderma Isolates (Optimum temperature for growth : 22 °C)

T.viride (Persoon ex S.F.Gray) Isolate 1

T.viride (Persoon ex S.F.Gray) Isolate 14 *

T.viride (Persoon ex S.F.Gray) Isolate 24

T.viride (Persoon ex S.F.Gray) Isolate 28

T.viride (Persoon ex S.F.Gray) Isolate 30

Unidentified *Trichoderma* Isolate 38

T.viride (Persoon ex S.F.Gray) Isolate 40 *

T.viride (Persoon ex S.F.Gray) Isolate 43

T.viride (Persoon ex S.F.Gray) Isolate 53

T.viride (Persoon ex S.F.Gray) Isolate 60 *

T.viride (Persoon ex S.F.Gray) Isolate 67

T.viride (Persoon ex S.F.Gray) Isolate 70

T.viride (Persoon ex S.F.Gray) Isolate 90

T.viride (Persoon ex S.F.Gray) Isolate 100

T.viride (Persoon ex S.F.Gray) Isolate 110

T.hamatum (Rifai) Isolate 150

Unidentified *Trichoderma* Isolate 170

Unidentified *Trichoderma* Isolate 190

T.polysporium (Rifai) Isolate 220

(* - indicates ten isolates selected for further study of antagonistic mechanisms Chapter 3, 4 and 5)

Basidiomycete cultures (Optimum temperature for growth : 25 °C)

Brown rot fungi

Neolentinus (=Lentinus) lepideus (Fr.:Fr:) Redhead and Ginns FPRL 7F

Gloeophyllum trabeum Karsten MAD 617

Postia placenta Fr. MAD 698

Antrodia carbonica P.Karst HHB 5104

White rot fungi

Trametes (=Coriolus) versicolor L. ex Fr: Pilat MAD 697

Phlebia brevispora Fr. HHB 7030

Irpex lacteus Fr. HHB 7328

All cultures were maintained in 3% (w/v) of Malt Extract Agar (Oxoid CM59) autoclaved at 121 °C for 15 min, poured into triple vented (Sterilin) petri dishes and incubated at the appropriate temperatures as mentioned above, in the dark. In all experiments, inoculum plugs (0.6 cm diameter) of the fungi were obtained from the margins of actively growing colonies.

2.2.2 Interaction Studies

Preliminary interaction studies were carried out with the above listed 38 ***Trichoderma*** isolates against two wood decay basidiomycetes, a brown rot fungus ***Neolentinus lepideus*** and a white rot fungus ***Trametes versicolor***. The interactions between the basidiomycetes and the ***Trichoderma*** spp. were carried out on two different media types. Medium 1 was 3% (w/v) Malt Extract Agar (MEA - Oxoid CM59), while medium 2 was a Low Nutrient Medium (LNM). Both media were autoclaved at 121 °C for 15 min prior to use. The low nutrient medium was selected because of its low nitrogen to carbon composition which is more representative of the nutritional status in wood. It was based on a nutrient solution formulated by Huttermann and Volger, 1973 and contained per liter of H_2O : 5g of D-glucose (Sigma G8270); 0.013g of L-asparagine (anhydrous) (Sigma A0884); 1g of KH_2PO_4; 0.3g of $MgSO_4$; 0.5g KCl; 0.01g of $FeSO_4$; 0.008g of $Mn(Ch_3COO)_2 . 4H_2O$; 0.002g $Zn(NO_3)_2 . 6H_2O$; 0.05g of $Ca(NO_3)_2 . 4H_2O$; 0.002g of $CuSO_4$, 0.008g of NH_4NO_3; 1% purified agar (Oxoid L28) was used for solidifying the medium (all chemicals were obtained from either BDH - British Drug Houses, Mercks Ltd., Glasgow or Fisons, Coatbridge, Lanarkshire, U.K., unless otherwise indicated).

Methods used for the fungal interaction studies were similar to those used by Rayner and Todd (1979). Mycelial plugs (0.6 cm diameter) removed from the growing margins of cultures of either ***N.lepideus*** or ***T.versicolor*** were placed at one side of a petri dish

containing each of the two media types and incubated at 25 °C and 70% relative humidity for 4 days. After this time, cores (0.6 cm diameter) removed from the margins of actively growing cultures of the ***Trichoderma*** isolates were placed at the opposite sides of the dishes and the plates were incubated in the dark at either 22 or 25 °C for up to 4 weeks (4 replicates/treatment). Two incubation temperatures were used to satisfy optimal temperature requirements of individual ***Trichoderma*** isolates.

Plates were examined on a daily basis to determine the outcome of interactions between the organisms and were assessed on the basis of whether: 1) either organism was overgrown by its competitor and the rate at which overgrowth occurred; 2) contact and overgrowth was accompanied by browning and lysis of the basidiomycete mycelium; 3) the basidiomycete was completely killed by the ***Trichoderma***. Lysis of the basidiomycete mycelium was confirmed by microscopic examination of samples from the zone of contact between the two colonies where the basidiomycete had been overgrown by the antagonist, cores were also plated onto malt extract agar containing 4ppm benomyl (0.8g of benlate in 1 liter of 50% ethanol; stock solution diluted 1:100 with distilled water before use) to test viability of the decay fungus. Benomyl selectively inhibits growth of ***Trichoderma*** allowing the regrowth of the basidiomycetes. Lack of growth of basidiomycetes from these cores after 3 weeks incubation at 25 °C on the selective media, indicated that the basidiomycete had been killed by the antagonist.

After preliminary studies, seven ***Trichoderma*** isolates which produced a killing effect on both media types and against both basidiomycetes were selected for further study. Three isolates that showed a lethal effect against just one basidiomycete or in one media type were also included. These ten isolates were used for further investigation of their antagonistic mechanisms against the selected basidiomycetes (Chapter 3, sections 1 to 4) and included four strains of ***T.viride*** (Persoon ex S.F.Gray), three strains of ***T.pseudokoningii*** (Rifai), a strain of ***T.aureoviride*** (Rifai), a strain of ***T.harzianum*** (Rifai) and an unidentified ***Trichoderma*** isolate 140, all of which are highlighted by an asterik in the list of organisms used.

2.3 Results - Interaction studies

During initial screening of the ***Trichoderma*** isolates a variety of reactions were produced as a result of the antagonism between the ***Trichoderma*** isolates and the basidiomycetes. The qualitative features included overgrowth of the basidiomycetes by the ***Trichoderma*** often, though not always, resulting in death of the basidiomycetes (Figure 2.1). In some cases a stalemate situation occurred where both antagonist and pathogen met but no subsequent overgrowth of the organisms was produced.

It was evident from these preliminary studies (Table 2.1) that more ***Trichoderma*** isolates exhibited greater target specificity towards ***N.lepideus***. Of those isolates which produced a lethal effect, this was more often directed against the brown rot rather than the white rot fungus. Within the ***Trichoderma*** isolates tested, both interspecies and interstrain variability in their lethal effects was evident eg. of the 36 isolates of ***Trichoderma*** used 17 were ***T.viride*** and their responses towards the basidiomycetes in the two media types failed to follow any specific pattern. The 4 isolates of ***T.pseudokoningii*** however showed a consistent lethal effect against both the basidiomycetes on both media types. Also the lethal effect against the brown rot fungi was considerably more successful on the malt medium than on the low nutrient medium.

Logistic regression analysis (Kleinbaum ***et al.***, 1988) of the interaction studies results was undertaken to determine if the lethal effect was dependent on the following three factors, ***Trichoderma*** species, medium used, and target decay organism. The model created with the three factors above and all their possible interactions indicate that exclusion of any factors or interaction resulted in a significantly worse ($p<0.02$) fit, indicating that all the factors are important in the outcome of the result, this is further explained below.

In the above statistical analysis, it is assumed at first that none of the three variables mentioned above are important, to obtain a constant or null model. Now in order to determine whether any of the variables had an effect on

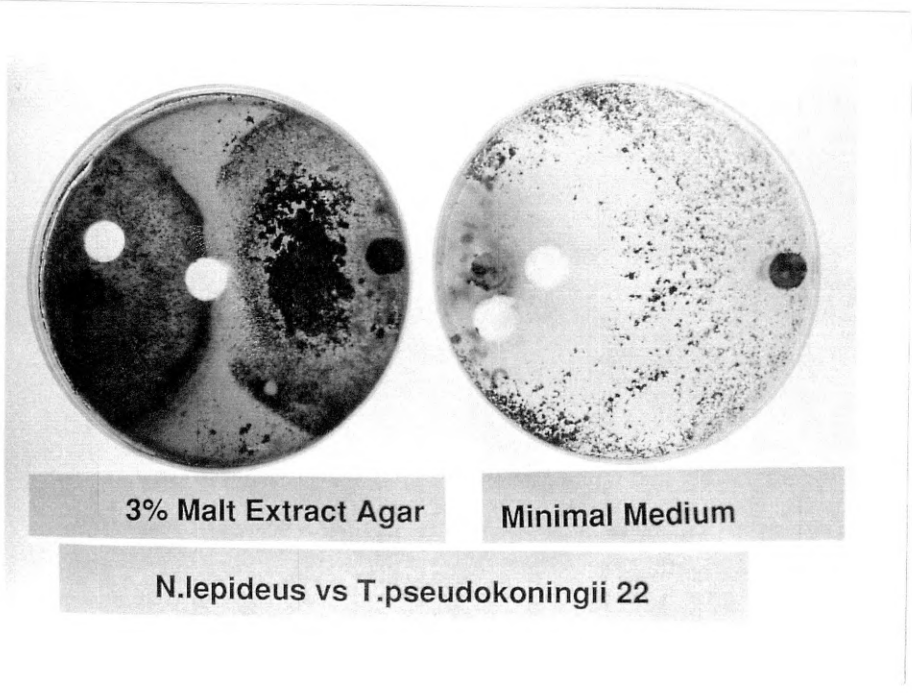


Figure 2.1 - Interaction plate between ***N.lepideus*** and ***T.pseudokoningii*** Isolate 22 on the two types of media showing a more severe overgrowth and lysis of the decay fungus on the malt medium than the minimal medium. The basidiomycete was inoculated on the left side of the plate and ***Trichoderma*** on the right side.

Table 2.1 Outcome of interaction studies between Trichoderma isolates and the two basidiomycetes. K=Complete death of decay fungus; S=Survival of decay fungus. (*) - Trichoderma isolates selected for further study of antagonistic mechanisms; and will be referred to from now on by their codes B1, B5, B11, B13, B14, B15, B16, R2, R7 and R10 as indicated against the respective Trichoderma spp.

Trichoderma isolates	Neolentinus lepideus		Trametes versicolor	
	3% Malt medium	Low Nut.medium	3% Malt medium	Low Nut.medium
T.aureoviride IMI91968 (B1) *	K	S	S	S
T. harzianum IMI206040	K	S	S	S
T.harzianum IMI335518	K	S	S	S
T.harzianum 25 (B11) *	K	K	S	S
T.polysporium IMI206039	K	S	S	S
T.polysporium 220	K	K	S	S
T.viride IMI24039	K	K	S	S
T.viride IMI49791 (B5) *	K	K	K	K
T.viride IMI335517	K	S	S	S
T.viride 1	K	S	S	K
T.viride 14 (R2) *	K	S	K	K
T.viride 24	K	K	S	S
T.viride 28	K	S	S	S
T.viride 30	K	S	S	S
T.viride 40 (R7) *	K	K	K	K

Trichoderma isolates	Neolentinus lepideus		Trametes versicolor	
	3% Malt medium	Low Nut.medium	3% Malt medium	Low Nut.medium
T.viride 43	K	S	S	S
T.viride 53	K	S	K	K
T.viride 60 (R10) *	K	K	K	K
T.viride 67	K	S	S	S
T.viride 70	K	K	S	S
T.viride 90	K	S	K	K
T.viride 100	K	S	S	S
T.viride 110	K	K	S	K
T.pseudokoningii 22	K	K	S	K
T.pseudokoningii 33	K	K	K	K
T.pseudokoningii 51 (B13) *	K	K	K	K
T.pseudokoningii 55 (B14) *	K	K	K	K
T.pseudokoningii 64 (B15) *	K	K	K	K
T.hamatum 150	K	K	S	S
Unidentified Trichoderma				
Trichoderma FYT sample	K	S	S	S
Trichoderma CCA sample	K	K	S	S
Trichoderma A(SH)	K	K	S	S
Trichoderma 38	K	K	S	S
Trichoderma 140 (B16) *	K	K	K	K
Trichoderma 170	K	S	S	S
Trichoderma 190	K	S	S	S

the results, the deviance of the constant model and deviance of the individual variables have to be compared. Regression analysis have been done including the following models - constant, constant+medium, constant+decay, constant+decay+medium, constant+species, constant+species+medium, constant+species+decay and constant+species+decay+medium. For example, the deviance of the constant model is 114.2 with 43 degrees of freedom, and the deviance for the model including medium is 108.6 with 42 degrees of freedom, then the difference in deviance is 5.6 with 1 degree of freedom. This difference can be used to estimate the importance of the medium in the model. If the medium is not significant then this difference in deviance value would be less than $Chi^2 (1)_{0.05} = 3.842$, i.e., the probability value indicating that this factor is not significant in the outcome of the results will be > 0.05, since the difference in deviance obtained here is greater than 3.842, hence medium is an important factor in determining the probability of lethal effect (kill) which is < 0.02. The importance of the other factors were analysed in a similar fashion (Table 2.2).

Deg. of freedom	Sets	Deviance	Diff. in Deviance	P(<)
43	Constant	114.2	0	
42	Medium(M)	108.6	5.6	0.02
42	Decay(D)	80.44	33.76	0.001
33	Species(S)	88.11	26.09	0.005
41	M x D	73.24	35.38	0.001
32	M x S	81.53	27.07	0.005
32	S x D	46.70	41.41	0.001
31	M x S x D	37.11	36.13	0.001

Table 2.2 - Regression analysis of the interaction results.

Effect of ***Trichoderma*** spp.

Trichoderma spp. had a significant ($p<0.01$) effect on the lethal outcome of the interactions although the significant interactions (M x S, $p<0.01$; S x D, $p<0.001$; M x S

x D, $p<0.001$; where M = media, S = species subgroup, and D = decay fungi) imply that the effect of ***Trichoderma*** spp. was also influenced by the media type and decay organism under attack.

Among the 36 ***Trichoderma*** spp. tested there were 6 different species and 7 unidentified ***Trichoderma***. ***T.pseudokoningii*** isolates showed the highest proportion of killing effect against both basidiomycetes on both media types followed by the ***T.viride*** isolates.

Medium effect

The type of medium used also has a significant ($p<0.02$) effect on the outcome of the interactions although the significant interactions (M x S, $p<0.01$; D x M, $p<0.001$; M x S x D, $p<0.001$) imply that the medium effect is dependent on the basidiomycetes under attack as well as the species of ***Trichoderma***.

It was evident that the lethal effect was more prevalent in the malt medium by all ***Trichoderma*** spp. against the brown rot fungi but less so against the white rot fungi. But those ***Trichoderma*** spp. that showed a lethal effect in the low nutrient media against both basidiomycetes are of particular interest because of their success in a medium that is nutritionally closer to the natural substrate, i.e., wood.

Effect of decay organisms

The decay organism under attack also has a significant ($p<0.001$) effect on the outcome of the interactions although again it is interlinked with the other factors (i.e., the ***Trichoderma*** spp. and the media type). This is evident from the results where the brown rot fungus were killed by almost all of the ***Trichoderma*** isolates on the malt medium but few were capable of killing the white rot fungus in the same medium.

2.4 Discussion

Trichoderma spp. have been screened as potential biocontrol agents of wood decay fungi due to their successful use in agriculture (Papavizas, 1985; Tronsmo, 1986; Campbell, 1989 a). Screening is an essential first stage in the development of any biological control agent and the results presented here illustrate the need for careful

selection of appropriate media for this purpose. Simplicity of the interaction studies has led to testing of fungal antagonists by cross inoculation of the antagonist and pathogen at opposite sides of agar filled petri dishes and the outcome of the interactions are observed. However, often little consistency is evident in the selection of media type and concentration between different researchers. This may well lead to controversial results by independent workers examining the same biocontrol isolates or may lead to expensive field testing of isolates which may not have passed through the preliminary testing procedure on an appropriate media.

Trichoderma are known to have the ability to grow over a wide range of nutritional conditions as evidenced by their ubiquitous nature (Papavizas, 1985). Due to such flexibility in their nutrient metabolism, analysis of carbon source utilisation patterns of ***Trichoderma*** isolates have been used by Manczinger and Polner (1987) as a method of species identification within the species aggregates of this problematic genus. Though this organism has low nutritional requirements for growth, the carbon, nitrogen and levels of other non-nutritional factors could possibly affect their success as an antagonist within specific environments (Danielson and Davey, 1973 a and b). For example they observed that in buffered media ***Trichoderma*** spp. grew best with L-alanine, L-aspartic acid, L-glutamic acid and casamino acids as sources of nitrogen. Growth on NH_4^+ nitrogen was consistently superior to growth on NO_3^- nitrogen. The best carbon sources were dextrose, fructose, mannose, galactose, xylose, ribose, trehalose and cellobiose. The ability to use melezitose, raffinose, sucrose and inulin as sources of carbon were used as a basis for taxonomic classification. Carter and Lynch (1991) have shown similar substrate dependent variation in the protein profile and antigens of ***T.harzianum***. Emphasising the influence of media nutrient composition on the synthesis and production of proteins by fungi. Among the variety of proteins produced there will also occur the inhibitory metabolites and lytic enzymes that play a role in the antagonistic mechanism of ***Trichoderma*** spp. And therefore exists a direct relationship between the nutrient composition of the growth media and the protein synthesis.

Fargasova (1992) studied the influence of various nitrogen sources on the growth, conidiation and pigment production of ***T.viride*** M-108 and its colour mutant strain. Nine

different nitrogen sources were tested and it was found that yeast extract and sodium nitrate proved to be the best nitrogen source for growth and conidiation of both parental and colour mutant strain of ***T.viride***. The best inhibitors of growth were adenine and ammonium sulphate. It was also found that higher concentrations of the nitrogen sources increased the intensity of conidiation. The influence of nitrogen concentrations at a molecular level was observed by Pitt and Bull (1982), while studying the influence of culture conditions on the physiology and composition of ***T.aureoviride***. They found that the mycelial RNA and protein concentrations in chemostat cultures of ***T.aureoviride*** were 30 to 40% lower under nitrogen limitation. Similar to the effects observed by the above authors with the influence of carbon and nitrogen ratios, it was apparent from the results of the interaction studies here that nutrient status of the interaction medium plays an important role in determining the outcome of the antagonist between ***Trichoderma*** and wood decay fungi.

It is crucial therefore during primary screening to devise a medium that closely represents the ***in vivo*** nutritional status in which the interaction of the pathogen and antagonist is to occur. The low nutrient media used here (Srinivasan ***et al.***, 1992 a, b) was hence selected to be more representative of the nutritional status within wood (C:N ratio is approximately 410:1 which falls within the range found within wood 350:1 to 1250:1, by Merrill and Cowling, (1966). Therefore the outcome of interactions in such a media might be expected to be similar to that occurring in the natural substrate i.e., in wood. Merrill and Cowling (1966) also noted that the nitrogen content of mycelia of some wood decaying fungi eg., ***Trametes versicolor*** and ***Fomes applanatus*** were related to the wood used as substrate. ***Trametes versicolor*** was also seen to adapt its nitrogen content to that of synthetic media. For this fungus, they noted similar nitrogen contents for mycelia grown on wood and on synthetic media with C:N ratios similar to those of wood. This again highlights the importance of use of appropriate media, as the physiology of both the antagonists and decay organisms can be greatly influenced by the substrate, which inturn affects the outcome of observations made in interaction studies.

The nutrients in the media apart from influencing the antagonistic responses (exhibited by a variety of modes of antagonism discussed in the later chapters), may also have a

role to play in the recognition of the decay fungi by the antagonists. For example, production of these 'hyphal sheath' which are known to be made up of carbohydrates, proteins, and lipids (Foisner ***et al.***, 1985 a, b) and have been proposed to play a role in the decay mechanism of wood by Green ***et al.***, (1989), may also be responsible for recognition by antagonists similar to 'lectin activity' found to be responsible for recognition between ***Rhizoctonia solani*** and ***Trichoderma*** species (Elad ***et al.***, 1983 a). Success of antagonists in inappropriate media may result in misleading assumptions with regard to target specificity of ***Trichoderma*** species. The results obtained here exhibit such effects very clearly, where the lethal effect that results in killing of the basidiomycetes on interacting with the ***Trichoderma*** isolates in the high nutrient media (MEA) fails to be reproduced in the nutritionally poorer media (LNM). It is also possible that the type of hyphal sheath produced in the LNM are more specialised and have a protective role that prevents recognition of the cell wall of the target pathogens, and therefore suppress the antagonistic effect of ***Trichoderma*** spp.

The results also stress the need for careful selection of ***Trichoderma*** isolates due to the variability that exists in the outcome of interactions, even within strains of the same species. ***T.viride*** was one of the dominant species among the 36 isolates tested. The isolates showed a high degree of consistency in antagonising the brown rot fungus in the malt medium, however this was not reproduced on the low nutrient medium. In their interactions with the white rot fungus however the same ***T.viride*** isolates failed to exhibit any pattern on either media. If the results obtained on the malt medium alone were considered, this could lead to the mis-interpretation that antagonists of this species have a target specificity towards the brown rot fungi. Therefore with use of inappropriate media, not only will the effective ***Trichoderma*** species be misinterpreted but also their target specificity. Such information is crucial for administration of particular strains of ***Trichoderma*** that can achieve effective control of specific decay fungi, like the brown rot of creosoted electricity distribution poles by ***N.lepideus*** (Bruce, 1983).

However all these factors are interrelated as is evident from the regression analysis of the results. These preliminary studies have not only emphasised the importance of

appropriate media for initial screening but also for further study of their antagonistic mechanisms. Despite the fact that many modes of antagonism that ***Trichoderma*** employ are known, the precise mechanism by which these antagonists control the decay fungi in wood has not been identified to date. It is essential to establish the mechanisms that are active ***in situ*** in order that appropriate methods can be developed for screening biological control agents. It is obvious from the results of the interaction studies that careful selection of media will be an essential part of the developmental process.

Chapter 3

Section 1

Soluble Metabolite Production

Chapter 3

Antagonistic Mechanisms

Section 1 - Soluble Metabolite Production

3.1.1 Introduction

Secondary metabolites produced by ***Trichoderma*** species have been extensively studied by many researchers, mainly because of their potential in biological control of plant pathogens. However, the taxonomic confusion surrounding the genus ***Trichoderma*** complicates interpretation of the earlier literature on secondary metabolites produced by these fungi. Thus those species involved in the production of gliotoxin (Weindling, 1934), gliotoxin and viridin (Brian and Hemming, 1945; Brian ***et al.***, 1946), and originally considered to belong to ***Trichoderma***, were subsequently identified by Webster and Lomas (1964) as ***Gliocladium*** species. The report (Moffat ***et al.***, 1969) describing the isolation of viridol from the culture filtrate of ***T.viride*** Pers. ex Gray contains a footnote stating that this isolate was the Weindling G-1 strain also described as ***Gliocladium fimbriatum*** Gilman and Abbott. A paper by Bu'Lock and Leigh (1975) refers to the biosynthesis of gliotoxin in ***T.viride*** (possibly a ***Gliocladium*** sp.) and another (Golder and Watson, 1980) to the biosynthesis of viridin in ***T.viride***. ***Trichoderma polysporum*** (Link and Pers.) Rifai, which has been subjected to several investigations and found to be a producer of antifungal cyclosporins (Traber ***et al.***, 1982), and ***Trichoderma sporulosum*** Hughes were both subsequently considered to be ***Tolypocladium niveum*** Bissett (Cannon, 1986).

It is possible that the above confusions may have contributed to some early views that ***Trichoderma*** spp produce few or no antibiotics (Cook and Baker, 1983) or that, at best, they are unreliable producers. The difficulty in classification seems to have frustrated some authors and engendered an attitude that since ***Gliocladium***, ***Trichoderma*** and ***Hypocrea*** are closely related genera a distinction between them is not critical. With the benefit of more rigorous classification, a detailed analysis of the metabolites known to

be produced by the species of the three genera was produced by Taylor (1986) (see Table 3.1), however this surprisingly shows little overlap between the genera.

Although Weindling and Emerson (1936) isolated antifungal substances from ***T.lignorum*** (later identified as ***Gliocladium*** by Webster and Lomas, 1964), Dennis and Webster (1971 a, b) were the first to describe the antagonistic properties of ***Trichoderma*** in terms of antibiotic production. They were able to show that ***Trichoderma*** species produce volatile and non-volatile compounds capable of inhibiting mycelial growth in a variety of fungi and that the production of antifungal substances varies with the isolate, even within the same species aggregates. In a sense this work marked the beginning of modern investigations on the role of antibiotics in the biological control of plant pathogens by ***Trichoderma*** species.

Taylor (1986) in his review on the secondary metabolites of the three genera ***Hypocrea***, ***Gliocladium*** and ***Trichoderma***, grouped the metabolic products of these fungi on the basis of their chemistry and particularly on their probable mode of biosynthesis. As the metabolites produced by the following species ***T.aureoviride***, ***T.viride***, ***T.harzianum*** and ***T.pseudokoningii*** are of particular interest in this study the corresponding metabolites produced by these species are listed in Table 3.1. The list was updated using a more recent paper published by Ghisalberti and Sivasithamparam (1991) on antifungal antibiotics produced by ***Trichoderma***.

Most of the knowledge on the antifungal role of ***Trichoderma*** metabolites is gathered from research with soil plant pathogens. ***T.harzianum*** Rifai is perhaps the most studied of the ***Trichoderma*** species for biocontrol and possibly the most effective. Although most reports in literature indicate that ***T.harzianum*** is effective in reducing disease caused by soil-borne plant pathogens (Chet, 1987; Papavizas, 1985) some variability in their effect has been reported (Wells and Bell, 1979; Maas and Kotze, 1987; Ghisalberti ***et al.***, 1990). Furthermore some of the early studies have failed to reveal the involvement of any typical antibiotics (Chet, 1987).

Producing organism	Metabolite name	m.p.(C)	References
Group I - Polyketide metabolites			
T.viride	Pachybasin	176*	Slater *et al.*, 1967
	Emodin	256-8*	Slater *et al.*, 1967
	Hypochrysophanol	205*	Moss *et al.*, 1975
T.pseudokoningii	Tartronic acid	158-60*	Kamal *et al.*, 1971
Group II - Terpenoid metabolites			
T.viride	Gliocladic acid (sesquiterpene heptelidic acid)		Itoh *et al.*, 1980
	Trichodermin (trichothecane sesquiterpene)	46	Godtfredson and Vangedal, 1965
	Viridin	245*	Brian *et al.*, 1946
T.pseudokoningii	Trichodermene A		Kamal *et al.*, 1971
	Pyrocalciferol	93-5	Kamal *et al.*, 1971
Group III - Non-polypeptide metabolites			
T.viride	Trichoviridin	102*	Tamura *et al.*, 1975
T.harzianum	Isonitrin D	55	Fujiwara *et al.*, 1982
Trichoderma spp.	Trichorin A	234-46*	Katayama *et al.*, 1977
	Trichorin B	212-4*	
Group IV - Polypeptide antibiotics			
T.viride	Trichotoxin	187*	Hou *et al.*, 1972; Bruckner *et al.*, 1979.
	Suzukacillin	250*	Ooka *et al.*, 1966; Katz, 1983
	Alamethicin		Brewer *et al.*, 1987
T.harzianum	Trichorzianine	253-4*	Bruckner *et al.*, 1984
	Harzianopyridone		Dickinson *et al.*, 1989

Table 3.1 - Secondary metabolites produced by ***Trichoderma*** species. (*) indicate those metabolites stable at 90°C (see section 3.1.2). References in the above table are as cited in Taylor, 1986; Ghisalberti and Sivasithamparam, 1990.

Some volatile antibiotics have been shown to play a role in the antagonism of these ***Trichoderma*** species, and these will be discussed later in section 3.2.1. Some polyketide and peptide metabolites produced by isolates of ***T.harzianum*** have been known to show inhibitory activity against the take-all fungus (***Gaeumannomyces graminis***), ***Rhizoctonia solani*** and ***Botrytis cinerea*** (Ghisalberti and Sivasithamparam, 1990; Almassi ***et al.***, 1991; Dickinson ***et al.***, 1989). These authors have also noted the degree of variation in the amounts and types of individual metabolites produced by the isolates. This variation in the production of metabolites may be attributed to the nutrient consistency of the ***in vitro*** growth media as realised by authors, Sierota (1977), Williams and Vickers (1986), Bushell (1989), Park ***et al.***, (1991).

Similarly there are numerous publications with regard to production of metabolites by other ***Trichoderma*** species including ***T.koningii***, ***T.hamatum***, ***T.viride*** and their inhibitory effect against a number of plant pathogens. However, there are few papers that deal with inhibition of basidiomycetes by ***Trichoderma*** metabolites. Sierota (1977) described the inhibitory effect of ***T.viride*** filtrates on ***Heterobasidion annosum***. Tokimoto ***et al.***, (1987) noted the increased levels of induced antifungal substances in cultures of ***Lentinus edodes*** by the attack of ***Trichoderma*** spp. Bruce ***et al.***, (1984) found that ***Trichoderma*** spp controlled the growth of wood degrading basidiomycete ***Lentinus lepideus*** and suggested that although water-soluble antibiotics were produced, and were effective against ***L.lepideus***, volatile metabolites alone may have been responsible for antibiosis. Bettuci ***et al.***, (1988) have explored the use of ***Trichoderma*** spp. and ***Gliocladium virens*** for control of wood-rotting fungi by soluble metabolites. All such work to date has however, involved use of media that do not in any way represent the nutritional status of the ***in vivo*** test material, i.e., soil or wood. Such studies may therefore not give a true representation of results to allow appropriate selection of potential biocontrol agents.

Researchers are becoming increasingly aware of the importance of selecting media that represent the ***in vivo*** nutrient composition, in order to truely assess the potential of biocontrol agents especially as production of a number of metabolites has been shown to

be related to nutrient stress as observed by Bushell (1989). Clearly many questions remain unanswered on the nature of the antifungal metabolites produced by ***Trichoderma*** species and their role in biocontrol. However recognising the importance of selecting appropriate media for such studies, can be considered a crucial step in revealing the answers to the biocontrol strategies of these organisms. The work reported in this section examines the importance of soluble metabolite production as an antagonistic mechanism of ***Trichoderma*** species and the influence of media composition.

3.1.2 Materials and Methods

The ten ***Trichoderma*** isolates selected on the basis of interaction results (see section 2.3; table 2.1) were grown in 150 ml broth cultures of each of the two media types (MEA and LNM autoclaved at 121 °C for 15 min) for 7 days at either 22 °C or 25 °C. The fungal mycelium was removed by filtration and the culture filtrate was sterilised by passing through a 0.45 µm sterile membrane filter (Whatman). The filtrates were placed in a 90 °C water bath for 2 hr to destroy enzyme activity. Ten ml of this filtrate was then added to an equal volume of strengthened agar (3% MEA, 3% purified agar), held at 50 °C, and poured into petri dishes to produce a solid medium for inoculation with the basidiomycetes. Cores (0.6 cm diameter) removed from the margins of actively growing cultures of ***N.lepideus*** or ***T.versicolor*** were inoculated in the centres of the plates which were then incubated at 25 °C. Controls were prepared by adding either uninoculated 3% malt extract (ME) (Oxoid L38) or LNM broth to the strengthened agar to replace the filtrates. Four replicate plates were set up for each test. Inhibition of growth of basidiomycetes was recorded as the difference in mean radial growth (measured from the edge of the core to the outer colony edge) of basidiomycetes in the presence or absence of fungal filtrates. These values were then used to calculate the inhibition of hyphal extension as a percentage of hyphal extension in the presence of the ***Trichoderma*** filtrates. Example - If radius of growth of basidiomycetes in the presence of the soluble metabolites is X and in its absence is Y, then % inhibition of growth of the basidiomycetes by the soluble metabolites of the ***Trichoderma*** is,

$$\% \text{ inhibition of growth of basidiomycetes} = \frac{Y - X}{Y} \times 100$$

3.1.3 Results

Figures 3.1.1, 3.1.2, and 3.1.3 show the growth inhibition caused by soluble metabolites from ***Trichoderma*** isolates in the two media types (MEA and LNM).

The results were analysed using an analysis of variance test. A model was used which considers all the factors involved in the experiment that may have a role to play in the outcome of the results. For example the percentage growth inhibition caused by the soluble metabolites is dependent on the following factors, the ***Trichoderma*** species (S), the media (M) and the decay (D) organism tested against. However these factors influence the results not only on their own but also as a combined effect, so the model takes into consideration all the factor combinations, i.e., S, M, D, S x M, S x D, M x D and S x M x D. Therefore the model formula will be $Y = \mu + S + M + D + SM + SD + MD + SMD + \text{error}$, where μ stands for the overall average and Y is the % growth inhibition value which is dependent on the above factors. A probability of < 0.05 (Table 3.1.3) signifies that factor or factors involved in the test have an important role to play in the outcome of the results.

Most isolates show greater specificity towards the brown rot fungus than the white rot fungus. This is illustrated more clearly by the mean values (Table 3.1.1). The values however are also dependent on the type of media used and the inhibition was found to be greater in the MEA than the LNM. However, the growth inhibition is not only dependent on the media and the target decay organism, but is also interdependent on the ***Trichoderma*** species tested as illustrated in Table 3.1.2. It is clear from the mean values that growth inhibition by most species is higher in the malt medium with the exception of subgroups 1 (***T.aureoviride*** species) and 2 (***T.viride*** species), but fail to reproduce this effect in the minimal medium. The interspecies variability in the degree of inhibition is also evident from the table 3.1.2, where the inhibition is highest with metabolites of ***T.pseudokoningii*** species in MEA and lowest with ***T.aureoviride*** in the same media.

Interstrain variability also occurs as illustrated by the individual growth inhibition values (Figures 3.1.1 and 3.1.2), e.g., ***T.pseudokoningii*** (B13) causes 100% inhibition of ***N.lepideus*** when grown in MEA, while ***T.pseudokoningii*** (B14) shows no inhibition of this fungus under the same test conditions. This is indicated clearly by the high standard deviation of the mean % growth inhibition values as in table 3.1.2. Analysis of variance of the ***Trichoderma*** spp., medium type, decay organism and their interactions (Table 3.1.3) shows that each individual variable has a significant effect on the degree of inhibition (S, $p<0.001$; M, $p<0.001$; D, $p<0.001$). Significant interactions of some of these factors imply that their effects are influenced by one or other eg., the ***Trichoderma*** spp. effect is affected by the media type (S x M, $p<0.001$). The success of a particular ***Trichoderma*** spp. however does not depend on the decay fungus under attack (S x D, $p<0.359$; S x M x D, $p<0.120$). Summarising, it is clear that the percentage growth inhibition of the basidiomycetes is dependent on the ***Trichoderma*** spp. subgroup and the medium type used but is irrespective of the individual decay fungi that is under attack. Therefore, it could be speculated that the inhibitory soluble metabolite is not specific to either of the decay fungi tested however its production is dependent on the media consistency.

Medium type	Decay Organisms	
	T.versicolor	N.lepideus
Malt agar	7.97 (17.80)	25.39 (30.19)
Low nutrient agar	0.10 (0.63)	4.69 (6.35)

Table 3.1.1 : Mean growth inhibition (%) of each basidiomycetes on the two media. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses.

Medium type	Trichoderma species subgroups				
	1	2	3	4	5
Malt agar	1.07 (1.77)	3.42 (4.18)	27.67 (23.8)	32.03 (37.4)	19.21 (11.56)
Low nutrient agar	2.21 (3.31)	3.49 (6.41)	0 (0)	0.63 (1.45)	5.64 (6.80)

Table 3.1.2 : Mean growth inhibition (%) of basidiomycetes on both media types by the ***Trichoderma*** species subgroups. 1 - ***T.aureoviride***; 2 - ***T.viride***; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii***; 5 - unidentified ***Trichoderma***. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses.

Factors	F ratio	Degrees of freedom	P
Species subgroups(S)	6.46	4,140	p<0.001
Medium (M)	24.66	1,140	p<0.001
Decay fungus (D)	14.63	1,140	p<0.001
Interactions			
S x M	9.15	4,140	p<0.001
S x D	1.10	4,140	p=0.035
M x D	4.97	1,140	p=0.027
S x M x D	1.87	4,140	p=0.120

Table 3.1.3 : Analysis of variance of growth inhibition (%) of basidiomycetes by soluble metabolites of ***Trichoderma***.

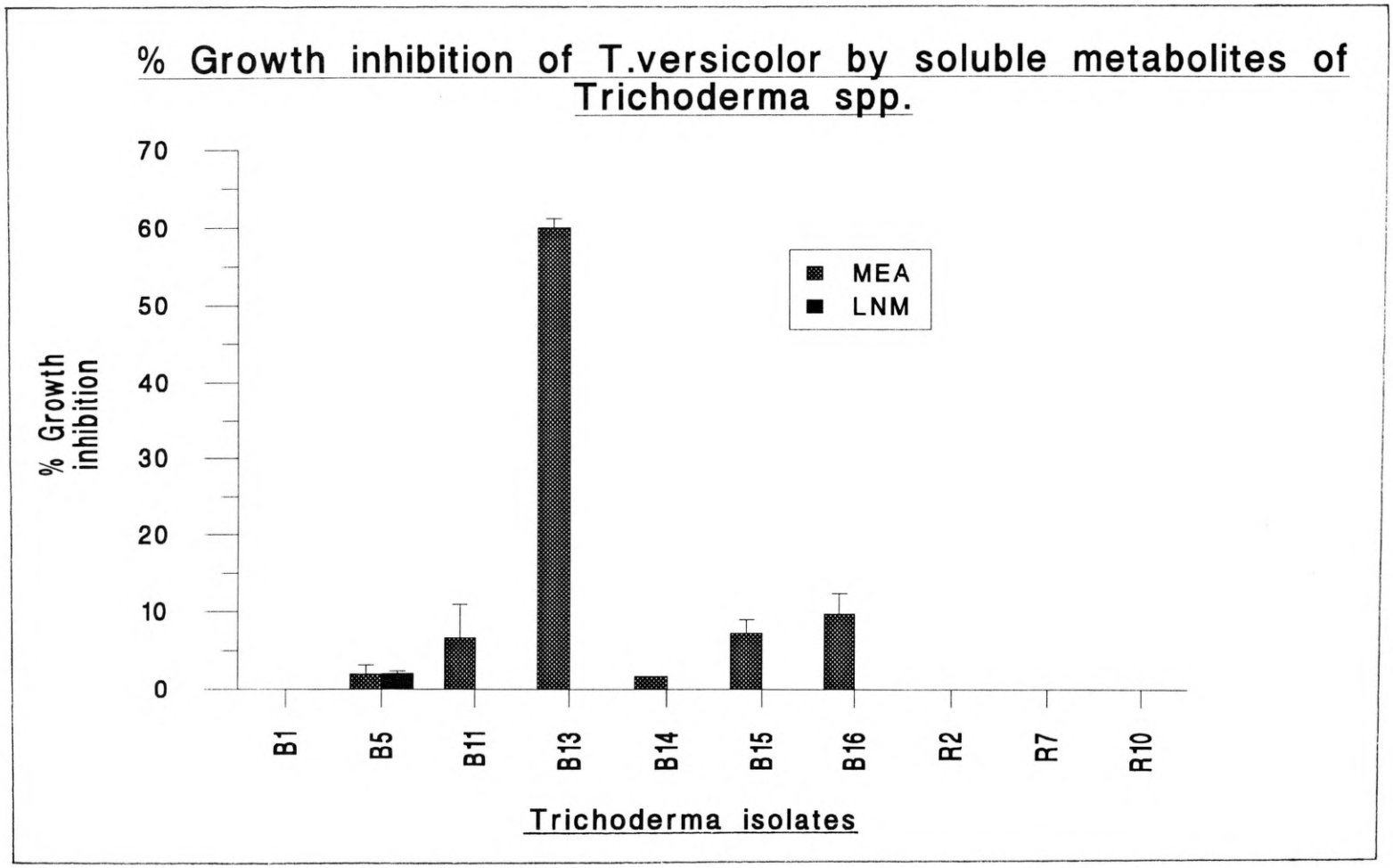


Figure 3.1.1 - Growth inhibition (%) of ***T.versicolor*** by soluble metabolites of ***Trichoderma*** isolates on the two media types. B1 - ***T.aureoviride***; B5, R2, R7, R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14, B15 -***T.pseudokoningii***; B16 - unidentified ***Trichoderma***.

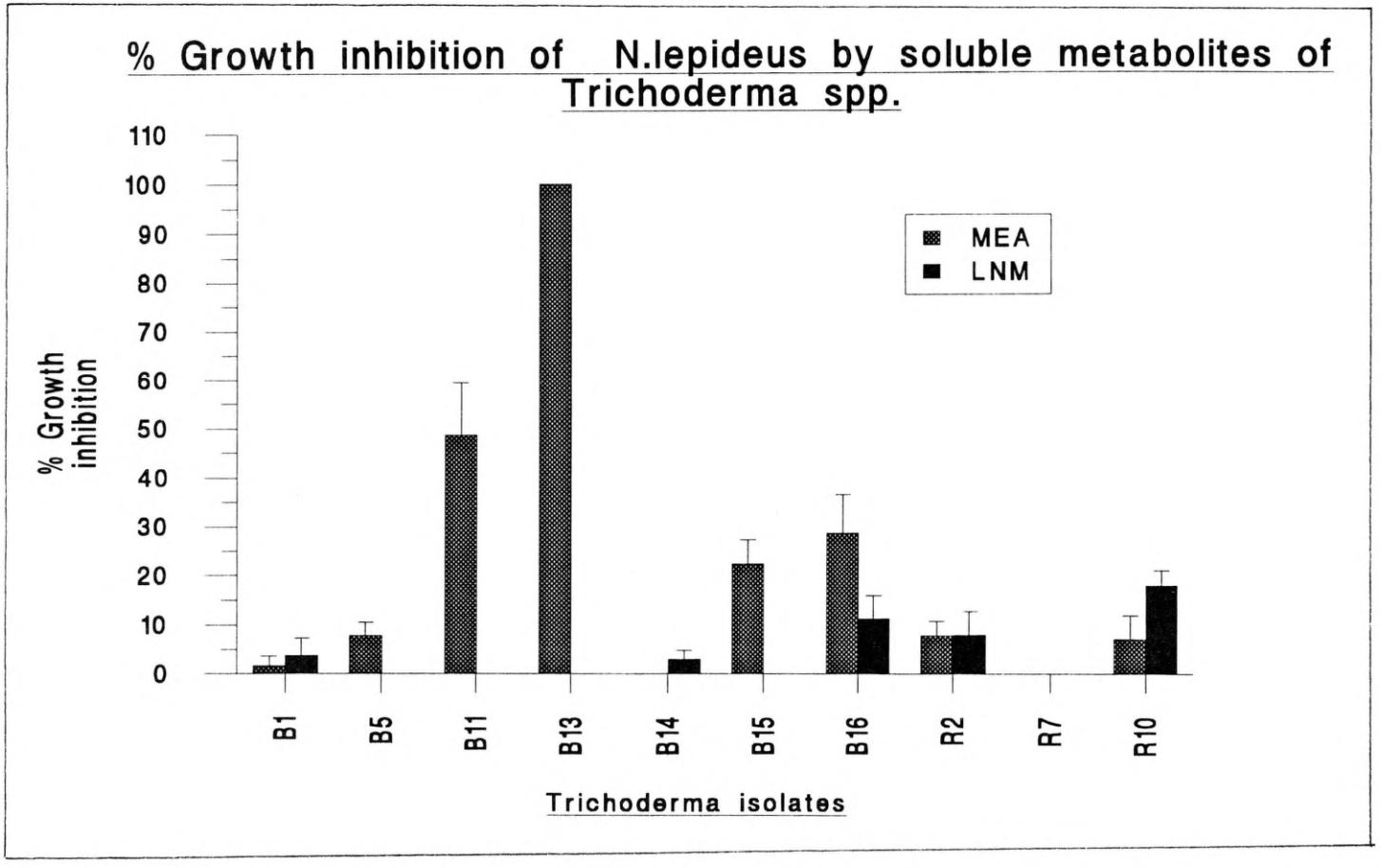


Figure 3.1.2 - Growth inhibition (%) of ***N.lepideus*** by soluble metabolites of ***Trichoderma*** isolates on the two media types. B1 - ***T.aureoviride***; B5, R2, R7, R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14, B15 -***T.pseudokoningii***; B16 - unidentified ***Trichoderma***.

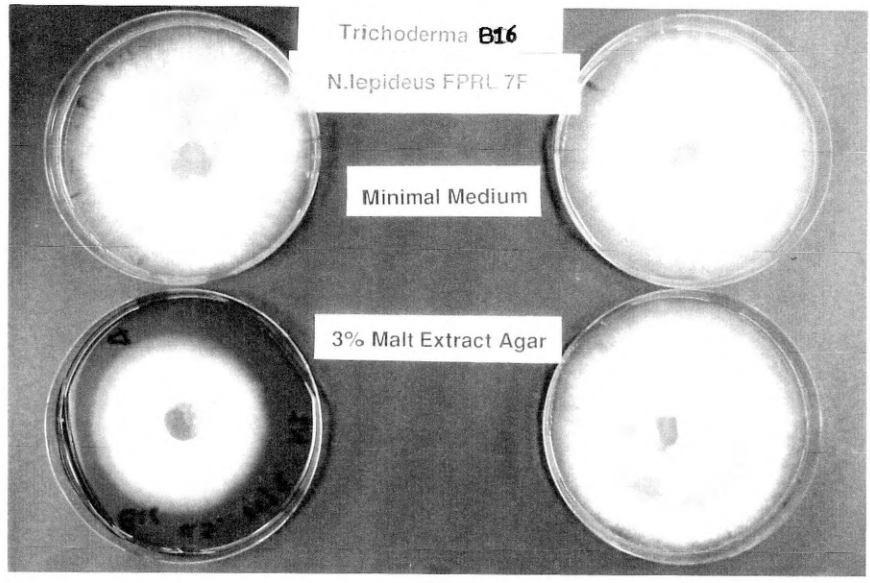


Figure 3.1.3 : Growth inhibition of ***N.lepideus*** by soluble metabolites of the unidentified ***Trichoderma*** B16 on both media types. Plates on the right are controls.

3.1.4 Discussion

Despite extensive research over the last 50 years on the capability of ***Trichoderma*** spp. to reduce the incidence of disease caused by soil-borne plant pathogens, and more recently on their application to inhibit wood degrading basidiomycetes, the mechanisms by which control is achieved are still not clearly understood.

One of the mechanisms that has been suggested to be involved in the biocontrol of many fungi is antibiosis. However it is apparent from the results presented here, that nutrient status of the growth medium is an important factor in determining the outcome of antagonistic responses with respect to production of inhibitory soluble metabolites (Srinivasan ***et al.***, 1992 a, b). Greater inhibition of the basidiomycetes was seen in the

malt medium with respect to production of soluble metabolites than in the low nutrient medium. Park ***et al.***, (1991) have reported the production of gliotoxin by ***Gliocladium virens*** as a function of source and concentration of carbon and nitrogen. It was observed that as the C:N ratio (glucose:phenylalanine) was increased from 18:1 to 80:1, the fungal growth rate decreased and this was directly related to decrease in concentration of gliotoxin produced. Similarly, Sierota (1977) observed the inhibitory effect of ***T.viride*** filtrates on ***Heterobasidion annosum*** in relation to some carbon sources. d-Xylose and d-glucose as carbon sources in the media gave the highest degree of inhibition when their filtrates were directly tested against ***H.annosum*** mycelium. The protection of pine wood blocks with filtrates was most effective when these were derived from media containing cellulose and d-xylose.

Apart from variations in the level of production of metabolites, the ***Trichoderma*** isolates in this study were also seen to exhibit target specificity towards the brown rot fungus ***Neolentinus lepideus***. This indicates that the ***Trichoderma*** can either recognise or access the target components of the brown rot fungi better than that of the white rot fungi or the latter has a better defence system that masks their presence. It has recently been reported that both brown and white rot fungi can produce hyphal sheaths. Foisner ***et al.*** (1985 a, b) and Messner ***et al.*** (1984 a, b and 1987) described in detail for the first time, using transmission electron microscopy, extracellular membranous structures (sheath) of brown- and white-rot basidiomycetes, and suggested a possible role for these structures in wood decay. Extracellular layers (hyphal sheath) enveloping vegetative hyphae of fungi are a common morphological feature (Bracker, 1967; Palmer ***et al.***, 1983). Hyphal sheaths are postulated to provide several functions for living hyphae; eg., 1) attachment to solid substrate, 2) a nutrient reserve, 3) reduce desiccation and nutrient loss, 4) provide protection against toxic chemicals, 5) facilitate wood degradation by storing or concentrating wood degrading agents, f) maintain a favourable moisture/pH environment for enzyme activity, and g) conditioning the substrate prior to enzyme action (Highley, 1987; Green ***et al.***, 1989 b). It is possible therefore that the protective hyphal sheath of the brown rot fungi is not produced in high amounts in the LNM, thereby permitting greater access to the cell wall than occurs in the white rot fungi. But since the inhibition of the brown rot is also comparatively higher in the MEA, it may be

speculated that the hyphal sheath produced by the white rot fungi are overall more protective than that of the brown rot fungi.

The design of the experiment however, examined only the effect of metabolites constitutively produced in either of the two media types. The high level of inhibiton in MEA may not therefore represent the inhibition of this particular pathogen which may occur when these two organisms are competing ***in situ*** in wood, where the presence of the decay fungus may act to induce the production of the antagonistic metabolites by the ***Trichoderma.***

Constitutive production of metabolites and induction of others by the presence of target organisms in ***Trichoderma*** was observed by Tokimoto (1987). Metabolites produced as a result of the presence of the target pathogen were named "mycoalexins" by these authors analogous to "phytoalexins" produced by plants as part of their defence mechanism. It is possible that ***Trichoderma*** isolates or their metabolites are capable of recognising or of being directed against the surface antigens of certain decay organisms more readily than others. Therefore the analysis of variance of the results (table 3.1.3) that show media type is more important rather than target organism in determining the level of inhibition is strictly because it is based on the response of the antagonist in each respective media type rather than against the target pathogens. The absence of any induction factor such as target cell walls may also account for the low response in the LNM. This lack of induction may be overcome in the MEA due to its high nutrients, however any metabolites produced may not necessarily still represent these which would be produced in the presence of the pathogen. Introduction of the target pathogen mycelium or cell wall material into the liquid culture media in which the ***Trichoderma*** is grown, may give a more accurate representation of the metabolites likely to be produced during interaction between the ***Trichoderma*** and its target in wood.

Tokimoto ***et al.***, (1987) had problems with ***Trichoderma*** and related fungi, as they attacked ***Lentinus edodes*** mycelia in bed-logs and therefore reduced the yield of the edible mushroom. They established that production of antibiotics like trichopolyns I and II by ***T.polysporum*** and production of lytic enzymes by other ***Trichoderma*** spp.

increased when they contacted the ***Lentinus*** mycelium (Tokimoto, 1982, cited in Tokimoto ***et al.***, (1987)) or with cell wall material of ***Lentinus*** lacking extracted proteinaceous material (Kitamoto ***et al.***, 1984, Tokimoto ***et al.***, (1987)). Presence of the pathogen mycelium may be a necessity if the antibiotic produced is specifically directed at the membrane of the pathogen, causing lysis like the trichotoxin A-40 (a membrane-exciting peptide) produced by a ***T.viride*** isolate (Irmscher ***et al.***, 1978). Therefore such metabolites may work on the basis of a trigger mechanism, and ***Trichoderma*** isolates may stimulate production only in the presence of the pathogen.

Tokimoto ***et al.*** (1987) also noted that the antifungal substances produced in culture by the ***Lentinus edodes*** differed before and after ***Trichoderma*** attack. It is possible that these components are part of the antigenicity or recognition signals of the decay fungi, that is picked up by the antagonists. The interaction outcome was also found to be dependent on the carbon source of the medium, i.e., the production of metabolites was greater in medium with glucose than with xylose. And there also seemed to be target specificity in the production of metabolites, though many different metabolites were detected in liquid culture of ***Trichoderma*** before inoculation of the decay fungi only certain ones were found to be produced in the presence of the decay fungi. Thus the above observations made by these workers highlight the complexity related to production of such metabolites, not only is their production dependent on the type of medium used but also on their target. Any antagonistic effect could therefore be by a single toxic metabolite or a multiplicity of components, the production of which are governed by complex regulatory mechanisms.

Researchers trying to reproduce antagonistic effects seen in liquid culture media in the laboratory in soil, have failed on numerous occasions (Williams and Vickers, 1986). The reasoning put forward for the lack of correlation is that, attempts to establish the presence in the soil of those metabolites produced in liquid culture have not been successful. It is possible that the organisms in liquid culture produce modified metabolites. The production of such modified metabolites needs to be established between MEA, LNM and wood. If this difference is the reason for varied results between MEA and LNM, then the results of LNM that is nutritionally closer to wood

may be speculated to be the true results ***in situ***. This therefore illustrates the importance of devising a media similar in nutrient consistency to wood for such experimentation. Also interesting is the observation for ***T.harzianum***, in which Ghisalberti and Sivasithamparam (1991) observed enhanced metabolite production on ageing. Since the metabolites are produced on ageing it is quite plausible that they are produced as part of the secondary metabolism of the fungus. The results obtained by Lumsden ***et al.*** (1990) would also appear to indicate that successful biocontrol action is associated only with a transient growth of ***T.harzianum*** during which there is no more increase in biomass.

This leads to the hypothesis that some of these biological control agents may have to be in a growth decline phase before they can produce high enough amounts of antibiotic metabolites. Nutrient stress in liquid culture would seem to approach more closely the conditions in which these organisms naturally find themselves. In competition with other organisms for limited nutrient supply, the producer of antibiotics would appear to have some advantage in ensuring its growth and survival. It seems reasonable to suggest that in terms of economy an organism would divert its metabolic effort to making antibiotics only when it is in competition for a substrate which it requires for growth. This situation was mimicked by Bushell (1989) who used a cyclic fed batch culture, where the substrate concentration was deliberately kept low. Wood has a low nutrient content and these are more irregularly distributed even than in soil, due to their redistribution in the processed timber. This redistribution of nutrients within wood is mainly due to evaporation of water during the drying of wood, which forces most of the nutrients to the surface (King ***et al.***, 1974). However, the essential carbon and nitrogen sources in the wood that are necessary for growth of the fungi still remain and it is important to include these carbon and nitrogen sources in any artificial media to mimic the nutrient consistency of wood especially since observations made by workers with soil emphasise the importance of selecting the correct media for study of metabolite production.

To complicate matters, apart from the media factor, and the decay organism under attack, each of the individual ***Trichoderma*** species and strains also show variation in their responses. In this study it was clear that ***T.pseudokoningii*** and ***T.harzianum***

species exhibited higher antagonistic potential in comparison to the other species in the MEA. However these species failed to reproduce this effect in the low nutrient medium. It is possible that as observed by Tokimoto ***et al.*** (1987), the presence of the target pathogen, i.e., the decay organisms is necessary for the production of any metabolites. Such interspecies and interstrain variability was also observed by Dennis and Webster (1971 a) in their study on production of non-volatile metabolites by ***Trichoderma*** spp.

A considerable amount of work has been reported on the antagonistic responses of ***Trichoderma*** spp. against plant pathogens, however given the complexity with which these antagonists operate, it cannot be assumed that the same metabolites that are directed against the plant pathogens are also responsible for inhibition of wood decay pathogens. Therefore further research still needs to be carried out to establish the specific inhibitory metabolites involved in the antagonism of wood decay fungi. However the first consideration in any such study must be the use of appropriate media, as has been shown clearly in this study. These antifungal compounds may have a significant role in the competitive saprophytic ability of these fungi in soil and wood, which is enhanced by their nonexacting nutritional requirements. It is important however to keep an open view about the effect of such metabolites. They might not necessarily be the sole agent responsible for the antagonism of the basidiomycetes by the ***Trichoderma*** spp. It is possible that these antibiotics weaken the basidiomycete cell walls (like, Trichotoxin A-40 and Alamethicin, the membrane damaging metabolites) and predispose them to parasitism, via the production of lytic enzymes like laminarinase and chitinase by themselves or by other strains. Thus also emphasising the importance of mixed culture antagonism. Where, if more than one antagonist is tested against a pathogen the antagonistic effect of one can be complemented by the other, for example, high production of soluble metabolite by one isolate may help in the first stages of antagonism followed by lytic effect of enzymes produced by another isolate in the same culture medium. It might also be an important consideration in terms of field application, to combine species or strains of ***Trichoderma*** with varied antagonistic capabilities. However by identifying and characterising the specific metabolites involved in the antagonistic process, such antifungal compounds may also be synthetically made for use as a wood preservative product, with numerous applications.

Chapter 3

Section 2

Volatile Antibiotic Production

Chapter 3

Antagonistic Mechanisms

Section 2 - Production of Inhibitory Volatiles

3.2.1 Introduction

In contrast to the wide interest in production of non-volatile antibiotics by ***Trichoderma*** species very little work has been carried out on the production of volatile antibiotics. Although the nature of these volatile antibiotics identified to date are relatively simple (eg., ethyl alcohol, ethyl acetate, octanol etc.) (Taylor, 1986) in comparison to the soluble metabolites, the huge variety of components, the low levels in which they are produced and their emission at different times during the life cycle of the antagonists makes their study a far more difficult task.

Again there is far more literature on effect of ***Trichoderma*** volatiles on plant pathogens than against wood decay basidiomycetes. However knowledge gained from such studies may help to understand their possible role in inhibition of growth of basidiomycetes. Dennis and Webster (1971 b) were one among the earlier pioneer workers to observe the production of volatiles in ***Trichoderma*** spp. and realised the variations in their production within isolates, and even within the same species aggregate. In their work they often observed that antagonistically active isolates were all associated with a "coconut" smell which had previously been noted by Bisby (1939) and Rifai (1964, 1969). Furthermore they commented that those isolates that caused an appreciable inhibition in the growth of ***Rhizoctonia solani*** after only a day of exposure were only isolates having the specific smell. The substance responsible for this smell has since been identified as 6-n-pentyl-2H-pyran-2-one (6PP) by several groups and its biological activity against a number of plant pathogens has been demonstrated (Merlier ***et al.***, 1984; Claydon ***et al.***, 1987; Ghisalberti ***et al.***, 1990).

The biological significance of the production of volatile metabolites by these fungi have interested mycologists for many years (Bilai, 1956; Hutchinson and Gowan, 1972;

Tamimi and Hutchinson, 1975). This has been firstly because of their simple chemical nature, they include compounds like ethyl alcohol, ethyl acetate, sec-butyl alcohol, isoamyl alcohol, octanol, octa-3-one, oct-1-ene-3-ol (Saito ***et al.***, 1981) and acetaldehyde (Dennis and Webster, 1971 b) and secondly because of the part that may be played by such metabolites in the sexual reproductive cycle of many plant pathogenic species responsible for diseases in some of the worlds's most important agricultural crops including ***Phytophthora*** spp. (Reeves and Jackson, 1972; Pratt ***et al.***, 1972)

During studies carried out with a number of ***Trichoderma*** species, Dennis and Webster (1971 b) observed that their volatile inhibitory effect against a number of plant pathogenic fungi, showed no specific pattern in their degree of inhibition against any of the specific pathogens. Some of the volatiles were even found to stimulate growth of the pathogens. Although there exists a variability in the results, certain isolates that show a high inhibitory effect can be developed by further genetic manipulation to better express their existing inhibitory effect. In most cases the volatile products of ***Trichoderma*** have been found to be solely fungistatic, however, Bilai (1956) and Khasenov (1962) (quoted in Dennis and Webster, 1971 b) reported that ***Trichoderma*** isolates could produce fungicidal volatiles.

During some earlier studies, gases from cultures of a strain of ***T.harzianum*** inhibited growth of ***Aspergillus niger*** Van Tieghem, ***Pestalotia rhododendrii*** Guba and several saprophytic bacteria (Hutchinson and Gowan, 1972). The only metabolites detected in significant amounts were carbon dioxide and ethanol and the authors concluded that these compounds were responsible for the activity. Tronsmo and Dennis (1978) however, later showed that carbon dioxide did not account for all the inhibition by volatile components from ***Trichoderma***. Claydon ***et al.*** (1987) examined two strains of ***T.harzianum***, each of which produced the characteristic coconut aroma and had previously been recorded as an effective antifungal agent (Lynch ***et al.***, 1984). The two strains were found to produce 6-n-pentyl-2H-pyran-2-one (6PP) and the dehydroanalogue, 6-n-pentenyl-2H-pyran-2-one. The pentyl analogue, the major product, showed potent inhibitory properties against a wide range of fungi and considerably reduced the rate of damping-off in lettuce seedlings by ***Rhizoctonia solani***.

This supported the earlier observation of Merlier ***et al.*** (1984) who found 6-n-pentyl-2H-pyran-2-one (6 PP), produced by a strain of ***T.harzianum***, to be inhibitory to ***Ceratocystis (=Ophiostoma) ulmi*** (Buism). Nannf. and ***Botrytis cinerea*** Pers. This compound exhibited a wide range of activity against sclerotia-forming pathogens and the take-all fungus (***Gaeumannomyces graminis*** var ***tritici*** Walker).

Most of the work undertaken to examine the effect of ***Trichoderma*** volatiles against wood decay basidiomycetes have been by Bruce ***et al.*** (1984, 1987 a, b). Mould inhabitants of creosoted wood were tested for volatile activity against ***Neolentinus lepideus*** and other basidiomycetes. Only ***Trichoderma*** species were found to release volatiles which produced a fungistatic effects on ***Heterobasidion annosum*** and both fungistatic and fungicidal effects on two strains of ***N.lepideus***. Other basidiomycetes were largely unaffected by volatile production (Bruce ***et al.***, 1984).

All of the above work however involved the use of malt extract media for evaluating the inhibitory potential of ***Trichoderma*** volatiles. This is not however representative of the true nutritional state of the test substrate, which is crucial in the study of such complex regulation of metabolites. The following work reported here enable the appreciation of importance of media composition in such studies. The role of volatile antibiotics as a sole antagonistic mechanism and its effect on different target pathogens was also examined.

3.2.2 Materials and Methods

Agar plates of each of the two media types (MEA and LNM (see section 2.2.2) autoclaved at 121 °C for 15 min) and inoculated centrally with cores (0.6 cm diameter) of each of the ten ***Trichoderma*** isolates and the two basidiomycetes. Plates containing basidiomycete inocula were covered with a semipermeable polythene membrane (MacKay and Lynn, Edinburgh, U.K.) and inverted over the plates containing individual ***Trichoderma*** isolates (the ***Trichoderma*** and superimposed basidiomycete were always on the same media type) and incubated at either 22 or 25 °C (four replicates/treatment). Appropriate controls for each medium consisted of plates of either basidiomycete on the respective media superimposed over uninoculated plates of the same medium. Inhibition

of growth of the basidiomycetes was recorded as in the soluble metabolite experiment, (section 3.1.2., i.e., the difference in mean radial growth of the basidiomycetes in the presence and absence of the ***Trichoderma***). These values were then used to calculate the percentage inhibition of hyphal extension of the basidiomycetes in the presence of the ***Trichoderma*** volatiles.

3.2.3 Results

Figures 3.2.1, 3.2.2 and 3.2.3 show the percentage growth inhibition of the two basidiomycetes caused by volatiles from the ***Trichoderma*** isolates. In the MEA the inhibition of the brown rot fungus, ***N.lepideus*** is greater than that of the white rot fungus, ***T.versicolor***, whereas the inhibition of the white rot fungus is greater than the brown rot fungus in the LNM. All these features are clearly evident from the mean values shown in Table 3.2.1. As seen with production of soluble metabolites the ***Trichoderma*** isolates again exhibit interspecies variability in inhibition by volatile antibiotic production (Table 3.2.1) e.g., ***T.aureoviride*** species give a mean growth inhibition of around 50% against ***N.lepideus*** whereas the ***T.pseudokoningii*** isolates give a mean of only 2% inhibition under the same conditions. Unlike the results for soluble metabolites, the levels of inhibition produced by the individual ***Trichoderma*** isolates within one species group do not show much interstrain variability. Table 3.2.2 shows the analysis of variance of the growth inhibition values. It is clear from the probability values that all factors i.e., ***Trichoderma*** species groups, decay organisms tested, and the media type, play an important role in inhibition of the basidiomycetes by volatiles (S, $p<0.001$; M, p,0.001; D, $p<0.005$).

It is also evident that all factors and interactions determine the level of inhibition (S x M, S x D, M x D, S x M x D, each have a $p<0.001$) and are influenced by one another. Therefore, it can be concluded that the inhibition of basidiomycetes caused by the volatiles is influenced by all three factors, the decay organism under attack, the media type used, and the species of the antagonistic ***Trichoderma***. Unlike the soluble metabolites where the decay organism under attack had no bearing on the level of inhibition, the volatiles seem to be more target specific to selected decay fungi.

		Trichoderma species subgroup				
Medium type	Decay species	1	2	3	4	5
Malt agar	T.versicolor	10.49 (3.11)	9.65 (7.39)	0.30 (0.61)	0.30 (0.56)	2.77 (3.24)
	N.lepideus	49.99 (7.56)	29.23 (17.0)	2.13 (2.47)	1.92 (2.92)	2.13 (2.83)
Low nutrient agar	T.versicolor	10.79 (2.88)	3.34 (2.58)	11.51 (3.42)	3.29 (2.11)	2.15 (1.18)
	N.lepideus	0.17 (0.34)	4.90 (5.49)	0 (0)	0.36 (0.87)	0 (0)

Table 3.2.1 : Mean inhibition (%) values of basidiomycetes in the two media types by the volatiles of ***Trichoderma*** subgroups. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses. 1 - ***T.aureoviride***; 2 - ***T.viride***; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii***; 5 - unidentified ***Trichoderma***.

Factors	F ratio	Degrees of freedom	P (<)
Species subgroups(S)	30.56	4,140	0.001
Medium (M)	33.03	1,140	0.001
Decay fungus (D)	8.27	1,140	0.005
Interactions			
S x M	20.84	4,140	0.001
S x D	10.07	4,140	0.001
M x D	48.24	1,140	0.001
S x M x D	10.06	4,140	0.001

Table 3.2.2 : Analysis of variance of growth inhibition (%) of basidiomycetes by volatiles of ***Trichoderma***.

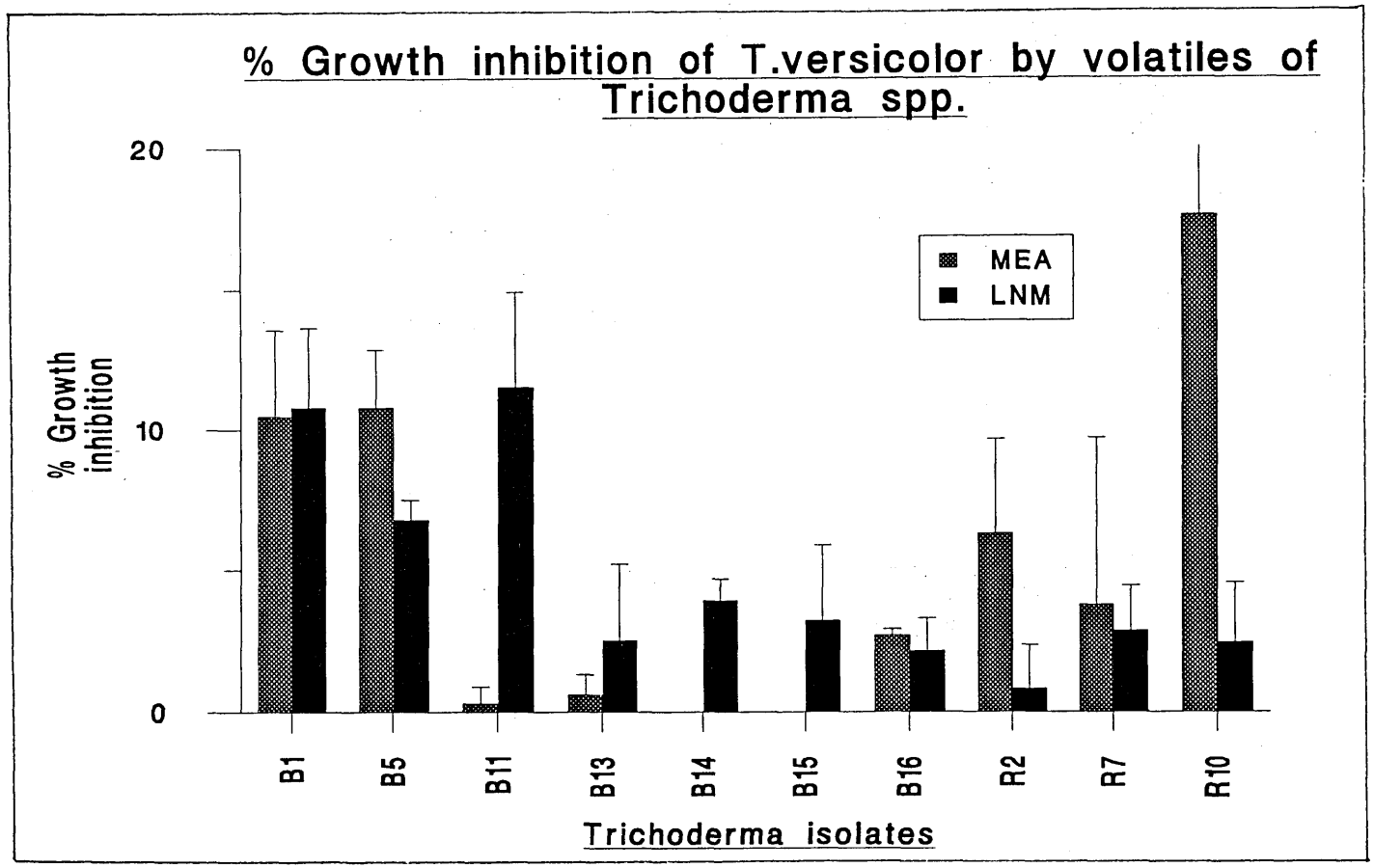


Figure 3.2.1 - Growth inhibition (%) of ***T.versicolor*** by volatiles of ***Trichoderma*** isolates on the two media types. B1 - ***T.aureoviride***; B5, R2, R7, R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14, B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***.

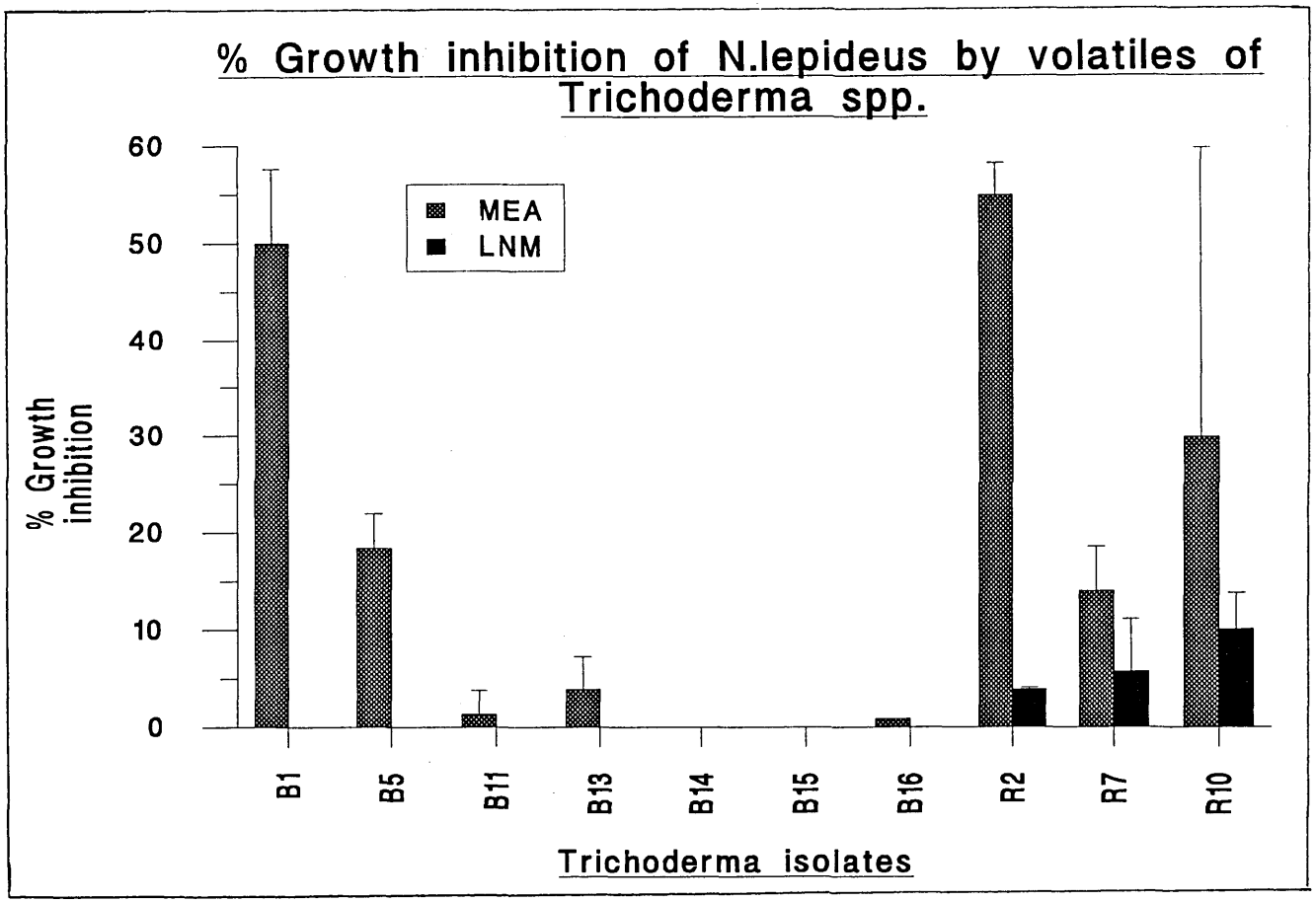


Figure 3.2.2 - Growth inhibition (%) of ***N.lepideus*** by volatiles of ***Trichoderma*** isolates on the two media types. B1 - ***T.aureoviride***; B5, R2, R7, R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14, B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***.

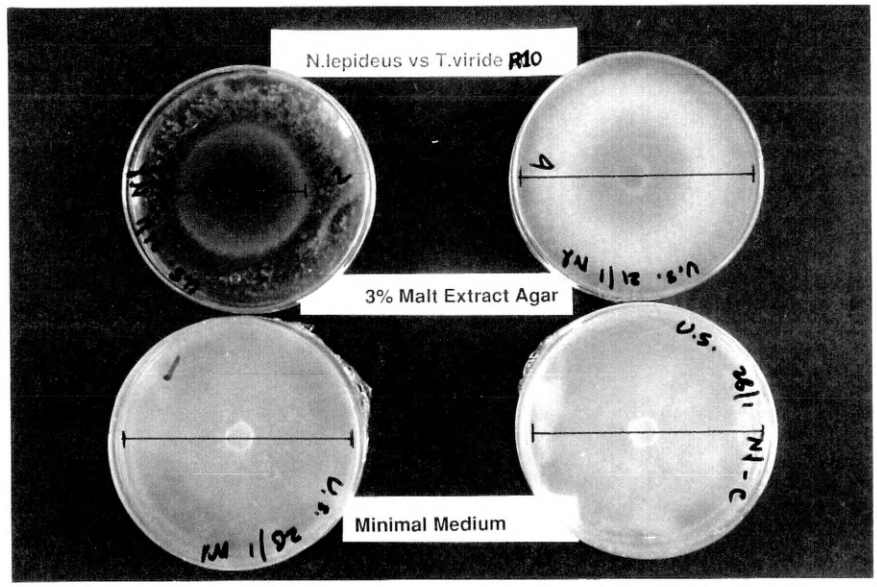


Figure 3.2.3 : Growth inhibition of ***N.lepideus*** by volatiles of ***T.viride*** R10 on both media types. Plates on the right are controls. Diameter of basidiomycete growth on the over plates is as indicated.

3.2.4 Discussion

Many workers have found it difficult to study the array of volatiles produced by ***Trichoderma*** species, due to the complex combinations in which they are produced. Though they are relatively simple in chemical composition, the degree of variability in their production within ***Trichoderma*** spp., against different pathogens at various times of their life cycle (Dennis and Webster, 1971 b) has made the task of identifying individual active compounds difficult. However, the effect of such volatile antibiotics against pathogens may be considerably more important in the ***in situ*** control of wood decay fungi than soluble metabolites due to their ability to diffuse further through the substrate in comparison to soluble inhibitory components.

From the results obtained here it is clear that production of inhibitory volatiles is not only dependent on the media type but also on the target organisms involved (Srinivasan ***et al.***, 1992 a, b). In MEA the inhibition of the brown rot fungus was generally higher than the white rot fungus, the situation was however reversed in the LNM, where the ***Trichoderma*** volatiles showed higher inhibition of the white rot fungus. This implies that volatiles produced only in the high nutrient medium result in lower inhibition of

growth of the brown rot fungus, however entirely separate volatile metabolites only capable of being synthesised in a LNM are responsible for the inhibition of the white rot fungus. This illustrates the importance of using an appropriate media for such studies, as results obtained in the MEA would have indicated that the ***Trichoderma*** species have a target specificity against the brown rot fungus. This target specificity could however be due to the protective effect of hyphal sheath produced by basidiomycete (Green ***et al.***, 1989 b) already discussed in section 3.1.4 with regard to soluble metabolites. The situation however seems to be reversed here in comparison to inhibition by soluble metabolites, as the brown rot fungi are inhibited less than the white rot fungi in the LNM. It is possible therefore that the hyphal sheath material produced in the LNM is providing better protection to the brown rot fungi against volatiles. Alternatively the particular target components on the white rot fungi may be detected better by volatiles than soluble metabolites thereby increasing inhibition due to better contact between the the antagonist volatiles and the components on the hyphal sheath.

As the volatiles produced in a high nutrient medium that is not representative of the natural substrate do not appear to be produced in the LNM their ability to inhibit the brown rot fungus in this medium cannot be considered to be of any practical significance for biocontrol ***in vitro***. However, the ***Trichoderma*** isolates (B1 and B11) that show inhibition of the white rot fungus in the LNM, may be of some importance. Interestingly these two isolates showed absolutely no inhibition of the white rot fungus in LNM by soluble metabolites. There seems to be a pattern in the expression of metabolites by ***Trichoderma*** spp. in that lower inhibition of the basidiomycetes by soluble metabolites is compensated with higher inhibition by volatiles and vice versa. It is however still curious that high inhibition of the brown rot fungus in the MEA is completely absent in the LNM and it is possible that due to very low nutrients the volatiles produced are limited and have no target specificity towards the brown rot fungi. But the limited range or quantity of inhibitory volatiles that are produced in the LNM are targeted against the white rot fungus. Such target specificity towards certain fungi was observed by both Dennis and Webster, 1971 b and Bruce ***et al.***, 1984, but both their studies involved the use of media such as malt extract which this study

indicates would give results that would not be repeated in a medium closer to the nutrient status of wood.

It was also evident from the results that there was a degree of interspecies variability in the level of inhibition against the basidiomycetes, as observed by Dennis and Webster, 1971 b. These authors used a number of strains of the following ***Trichoderma*** species, ***T.hamatum***, ***T.koningii*** and ***T.viride*** and noted that volatile antagonistic effect against the plant pathogen ***Rhizoctonia solani*** were both variable among and between species and strains of ***Trichoderma***. Interstrain variability however was not as evident in the observations made in this study. Dennis and Webster (1971 b) noted that there was also a certain degree of variation in the inhibition levels at different stages of ***Trichoderma*** growth. It is evident from the results obtained here that inhibition of basidiomycetes by most ***Trichoderma*** isolates is related to age of growth. In most cases it was clear that the inhibition of growth of the basidiomycetes was pronounced only after the fifth day of exposure to the antagonist until which time they had followed the growth rate of the controls very closely. Assuming the inhibitory volatiles are secondary metabolites they will only be produced after the growth period of the antagonist and therefore their effect will only be seen at that stage of growth.

Such differences in production of volatiles related to age of mycelium was also observed by Gervais and Sarrette (1990). The production of 2-heptanone, "cheese aroma" by ***Trichoderma viride*** cultivated on agar media was evaluated using headspace gas chromatographic analysis. An apparatus intended to measure the aroma production of different areas of a mycelial colony was set up. The study of the aroma production of these areas showed that the production values differed greatly and were dependent on the age of the colony and that volatile production from any one locus evolved with time. It was shown that mycelial aroma production was maximum when mycelia were about 3.5 to 6.5 day old, and this concurs with the inhibitory effects seen during this study.

There also seems to a correlation between the "coconut aroma" from the MEA culture media and the inhibitory effect of the ***Trichoderma*** spp. as observed by Merlier ***et al.*** (1984), Claydon ***et al.*** (1987) and Ghisalberti ***et al.*** (1990). These workers found that

this aroma was due to the compound 6-n-pentyl-2H-pyran-2-one (6PP) which is thought to be responsible for the inhibitory effect against selected plant pathogens. In the experiments carried out here it was found that the coconut aroma was only found in the MEA cultures and was absent in the cultures grown on LNM. And there was a definite correlation between the isolates that exhibited this smell in the MEA and higher inhibitory effect against the basidiomycetes. However, this effect was not evident in the LNM, and there were a few isolates that exhibited equally high inhibition level in this medium without the production of any coconut odour. This implies that perhaps these compounds may not have a specific role in inhibition of the basidiomycetes. It also illustrates the false interpretation of results due to use of inappropriate media. Though the coconut aroma is related to inhibition of basidiomycetes in the MEA, as this does not occur in the LNM which is closer in nutrient consistency to wood the results in the former media are of limited significance with respect to biocontrol.

It is possible that the volatiles like the soluble metabolites when produced may weaken pathogens and predispose them to the rest of the antagonistic strategies, including enzymes by other strains or species of ***Trichoderma***. In the hyphal interaction study of ***T.harzianum*** and ***T.polysporum*** with wood decay fungi by Murmanis ***et al.*** (1988 b), these authors observed that during the mycoparasitic interaction the hyphae of the decay fungi were void of cytoplasmic contents whether or not ***Trichoderma*** were present in the immediate vicinity. As the ***Trichoderma*** isolates used in their study did not produce any soluble metabolites as confirmed by agar plate testing, they speculated that denaturation of cytoplasmic contents observed in the hyphae of the basidiomycetes some distance away from the ***Trichoderma*** could be due to volatile components with inhibitory properties, that can affect organisms remote from the sites of production. It is reasonable to assume such a strategy with the results obtained with the isolates in this study as the volatiles only exhibited a fungistatic effect rather than a fungicidal effect against the basidiomycetes, therefore it is fair to assume that they do not comprise the sole antagonistic mechanism involved in elimination of the wood decay fungi. However, in previous work done by Bruce ***et al.*** (1984) on the effect of ***Trichoderma*** volatiles against a range of wood decay fungi, these authors observed both fungistatic and fungicidal effects. Considering the fungistatic effect of the volatiles, it is important to

realise that this could be a paramorphogenic effect. It has been observed by Skone and Dixon (1981) that certain carbon sources like sorbose caused a change in the fungal morphology while the biomass was unchanged in fungi like ***Ceratocystis adiposa***. This visual decrease in diameter of growth is compensated by increased production of aerial mycelium, thereby indicating the need to monitor the extent of biomass production. Substrates or compounds that stimulated such an effect were given the name paramorphogens. Though no unusual carbon sources were included in the LNM in the study of volatile antibiotic production, it is possible that the volatiles produced may show such paramorphogenetic effect against the wood decay fungi. This effect however cannot play any such role if the growth of basidiomycete is inhibited 100% by volatiles as observed by Bruce ***et al.*** (1984) or is likely to account for some of the higher percentage inhibition values recorded in this study.

It is clear from the above study that there is a complex regulatory mechanism involved in the production of these volatiles and selection of an appropriate media for such studies is important in determining the activity of any volatiles produced. Also since the volatiles are highly targeted against certain basidiomycetes or certain basidiomycetes are better at protecting themselves from such metabolites, any conclusions drawn with regard to inhibiton by volatiles should be preceded by careful experimentation. Also if the age related production of volatiles is repeated during colonisation of the wood substrate by ***Trichoderma***, mycelia at different stages of growth would protect themselves by emitting either volatiles or any other inhibitory component against other competitive fungi. However, there may be no fixed relationship between the physiological state of the mycelial tips at different stages of growth invading a substrate, and they might be responding on an individual basis to the signals that they pick up at their individual areas of colonisation. Metabolites or volatiles may therefore be produced as a individual response by hyphal tips rather than a whole colony reaction as measured in this study.

Knowledge gained with regard to the chemical consistency and mechanism of inhibiton by potent volatiles may be used to design volatile protectants for wood that are less toxic than the volatile chemicals that are already in use eg., Chloropicrin, Vapam. Such

bioprotectant volatiles may be used for more specific purposes like spraying of smaller confined indoor areas where the human toxicity of other volatile fumigants would create difficulties. However this can only be achieved if the exact chemical composition of the volatiles can be identified. The simultaneous distillation-extraction (SDE) system by Likens and Nickerson (1964) and modified by Godefroot, Sandra and Verzele (1981) is one of the most popular methods currently used to isolate volatile components from a matrix prior to gas chromatographic analysis (Maignail ***et al.***, 1992; references above as cited in this paper). Other methods like multisorbent thermal desorption/gas chromatography/mass selective detection methods developed by Heavner ***et al.*** (1992) for determination of target indoor volatile organic compounds in indoor air can also be modified to monitor volatiles produced by fungi. Though experimental facilities exist to determine the volatiles responsible for inhibitory effect, the complex varieties of volatiles that are produced on different media types and against different target organisms makes this a complex time consuming task.

Chapter 3

Section 3

Lytic Enzyme Production

Chapter 3

Antagonistic Mechanisms

Section 3 - Lytic Enzyme Production

3.3.1 Introduction

Parasitism is only one out of three main antagonistic relationships between micro-organisms - the other two being antibiosis and competition (Chet, 1987). When a parasitic fungus attacks a pathogenic fungus in a biotic system, it can be considered as a mycoparasite (Cooke, 1977).

During the seventies researchers did not realise that parasitism could indeed serve as an effective tool for biological control. During the last decade however, with increasing research in this field, and accumulation of data on the efficacy of mycoparasites as efficient biocontrol agents (Baker, 1987 b) this attitude has changed. There are several examples of this phenomenon and as far back as 1957, Tribe (1957) discovered that ***Coniothyrium minitans*** directly attacked the sclerotia of ***Sclerotinia trifoliarum***. Another effective mycoparasite is ***Ampelomyces quisqualis*** which invades powdery mildews (Sztejnberg ***et al.***, 1988). Lifshitz ***et al.***, 1984 found a new species ***Pythium nunn*** capable of causing lysis to germinating sporangia of ***Pythium ultimum*** in soil. Paulitz and Baker (1988) suggested that the mechanism involved in this system may be competition derived from ectoparasitic activity. However most of the work related to mycoparasitism since then has been carried out with ***Trichoderma*** spp.

The genus ***Trichoderma*** contains many species that are noted for their capacity to parasitise other fungi and to act as biocontrol agents. ***Trichoderma harzianum*** is the species most frequently reported to be an effective antagonist against plant pathogens. Its host range includes ***Sclerotium rolfsii*** (Wells ***et al.***, 1972), ***Rhizoctonia solani*** (Hadar ***et al.***, 1978), ***Pythium aphanidermatum*** (Sivan ***et al.***, 1984) and many others.

Mycoparasitism, a direct attack of one fungus on another has been reviewed in a very thorough way by Baker (1987 b). Many research reports deal with mycoparasitism as it relates to biological control (Baker and Cook, 1974; Chet, 1987). Interactions between mycoparasites and their target fungi occur in sequential but overlapping phases: target location by chemotrophic growth; recognition; attachment; penetration with aid of lytic enzymes and nutrient acquisition. The presence, duration and importance of each phase depend on the fungi involved; whether the mycoparasite is biotrophic (causes little harm to host) or necrotrophic (kills the host in advance or immediately on contact); the target organism being attacked; and the nature of the habitat and its prevailing environmental conditions. The successive biochemical events and fungal products involved in this phenomenon are as follows:

a) Chemotrophic growth - A positive chemotrophism is a directed growth towards a chemical stimulus. In 1981 it was found that ***Trichoderma*** can detect its host from a distance (Chet ***et al.***, 1981). The mycoparasite begins to branch in an atypical way and these branches grow toward the pathogenic fungus. Apparently ***Trichoderma*** grows according to a chemical gradient - however, no specific stimuli other than amino acids and sugars have yet been detected (Chet, 1990 b). It is not clear, therefore, if this phenomenon is specific to certain hosts. The above author also noted that although chemotrophism may have some advantage for the antagonist it is not an essential step for mycoparasitism. Chemotactic responses in host-parasite relationships were previously found in lytic bacteria (Chet ***et al.***, 1971) or nematode trapping fungi (Jansson and Nordbring-Hertz, 1979)

b) Recognition - In all experiments dealing with ***Trichoderma*** (Chet, 1990 b) it was found that the antagonist was rather specific and attacked only a few fungi. This led to the hypothesis that there is a molecular basis for such specificity which may depend on recognition. Among factors mediating the intercellular recognition between cells, lectins apparently play an important role.

Work done by Elad ***et al.*** (1983 a) showed that a lectin present in ***Rhizoctonia solani*** hyphae binds to galactose sugar residues on ***Trichoderma*** cell walls. This agglutinin

may play a role in prey recognition by the predator. Since many isolates of ***T.harzianum*** can parasitise ***Rhizoctonia solani*** the system studied by Elad ***et al.*** (1983 a) did not enable the study of specificity of different ***Trichoderma*** isolates. Barak ***et al.*** (1985) studied the role of lectins in the interaction of ***Trichoderma*** with another basidiomycete ***Sclerotium rolfsii***. This plant pathogen produced a lectin in solid and liquid media. Extracts of the fungus as well as the culture filtrate, agglutinated certain gram-negative bacteria and yeasts, but not human red blood cells. D-Glucose, D-mannose and several of their derivatives specifically inhibited the agglutination of cells of ***Escherichia coli***. Their study tested the possibility that recognition between the host and the ***Trichoderma*** isolate is responsible for specificity. Indeed, ***Sclerotium rolfsii*** lectin was found capable of only agglutinating the conidia of ***T.hamatum***, shown to attack this fungal host, and not the other tested ***Trichoderma*** isolates (Barak ***et al.***, 1985), even though all of them excreted lytic enzymes (Elad ***et al.***, 1982). Barak and Chet (In Chet, 1990 b) found that production of both lectin and 1,3-glucan, by the soilborne plant pathogenic fungus ***Sclerotium rolfsii*** is strongly affected by the chitin synthetase inhibitors polyoxin-D and nikkomycin. It appears, therefore, that this lectin is a glycoprotein (Barak ***et al*** 1985; Chet, 1987). The lectin may therefore play a role in recognition, which is apparently one of the factors involved in specificity.

c) Attachment - After recognition the hyphae of ***Trichoderma*** attaches to its host. When the mycoparasite reaches the host, its hyphae often coil around it or attach to it by forming hook-like structures (Harman ***et al.***, 1981). In the first case the attachment is carried out due to the formation of hook-like structures by ***Trichoderma. T.hamatum*** produced appressoria at the tips of short branches (Harman ***et al.***, 1981). This attachment is apparently the last step followed by the activity of the lytic enzymes. Earlier in 1971(c), Dennis and Webster studied hyphal interactions between ***Trichoderma*** and many other test fungi, where they observed similar coiling patterns as described above. They also observed that vacuolation, coagulation of cytoplasm and sometimes bursting of hyphae were induced by the antibiotic-producing strains.

d) Lytic enzyme activity - ***Trichoderma*** spp. are known for their ability to degrade fungal cell walls (Chet, 1987; Baker, 1987). This phenomenon is known to occur mainly

due to the excretion of the extracellular enzymes β-1,3-glucanase (laminarinase) and chitinase (Elad *et al.*, 1982).

β-1,3-glucanases represent a wide group of enzymes acting on diverse β-linked glucans such as β-1,3 and β-1,4-glucans. These glucans are widely distributed and often occur as structural components of cell walls or storage polysaccharides. The structure of the glucan backbone and the nature of substitution residues are highly variable (McCleary and Matheson, 1986). In plants, production of β-1,3-glucanases (laminarinases) and β-1,4-glucanases (cellulases) have been reported under normal developmental conditions and under stress. Glucanases acting on β-(1,3)(1,4)-glucans have also been detected in germinating grains of barely (Hatfield and Nevins, 1987). In many filamentous fungi, enzymes can either hydrolyse β-1,3-glucans in an exo or in an endo manner (Rapp, 1989). The former release only D-glucose as the end product of β-1,3-glucan while the latter produce a mixture of laminaridextrins with glucose as a minor product. Since β-1,3-glucans occur in both fungi and higher plants, fungal β-1,3-glucanases are thought to be important in the intracellular mobilisation of food reserves and in the extracellular hydrolysis of plant remains. They are also thought to play a role in their own growth cycle, as their walls are also made up of glucan and chitin (Chester and Bull, 1963; Webster, 1980). These enzymes are common in fungi, being detected in the culture filtrates of 96% of the organisms tested by Reese and Mandels (1959) in shake flasks and the enzyme is reported to be constitutive. Detection of β-1,3-glucanase activity is primarily determined by hydrolysis of laminarin (isolated from an alga, ***Laminaria digitata***) using a reducing sugar colorimetric assay or recently, after native polyacrylamide gel electrophoresis (PAGE) and iso-electric focusing (IEF) (Pan *et al.*, 1989). Detection of β-1,4-glucanases has been reported after IEF using carboxymethyl (CM) cellulose as substrate, stained with congo red or iodine (MacKenzie and Williams, 1984), or p-aniside (Eriksson and Pettersson, 1973, as cited in MacKenzie and williams, 1984). Detection of cellulase has also been reported after sodium dodecyl sulfate (SDS)-PAGE with CM-cellulose as substrate in addition to mixed β-(1,3)(1,4) barely glucan stained with congo red (Schwarz, *et al.*, 1987). More recently Cote *et al.* (1989) have developed a β-1,3-glucanase assay in native PAGE in gels containing laminarin as substrate stained with aniline blue.

Chitin a β-1,4-linked polymer of n-acetylglucosamine, is a major component of the cell wall of many fungi (Austin *et al.*, 1981; Peberdy, 1990). The enzymic degradation of chitin by micro-organisms occurs in two consecutive steps: first the hydrolysis by chitinase to oligomers, mainly dimers, followed by the degradation to free n-acetylglucosamine by chitobiose. The monosaccharide released can then be metabolised by micro-organisms (Jeuniaux, 1966). These enzymes are widely distributed in nature and have been detected in bacteria and fungi (Cabib, 1987). Fungal chitinases are involved in gross autolysis associated with the release of spores and stipe elongation in some basidiomycetes (Iten and Mantile, 1970) and autolysis of mycelium in cultures (Isaac and Gokhale, 1982). They may also have a nutritional role (de Vries and Wessels, 1973) and a morphogenetic function in hyphal growth and differentiation of fungi (Bartnicki-Garcia, 1973; Burnett, 1979; Wessels, 1984). In recent years several workers have suggested that chitinase plays a role in the mycoparasitism by the biocontrol fungus ***Trichoderma*** against fungal pathogens which is discussed in detail below. As with the laminarinases, chitinase activity has also been detected after PAGE.

After attachment and coiling of the antagonist around the phytopathogenic fungus, lysed sites and penetration holes were seen on the hyphae (Chet, 1990 b). In the presence of cycloheximide, antagonism was prevented and enzymatic activity was diminished (Elad *et al.*, 1983 b). Lynch (1987) and Ridout *et al.* (1988) supported these data by reporting that ***T.harzianum*** was able to penetrate hyphae of ***Rhizoctonia solani.*** It was noted that lytic enzymes were also found to be active in soil.

Sivan and Chet (1986) found that in ***in vitro T.harzianum*** failed to parasitise colonies of ***Fusarium oxysporum*** f. sp. ***vasinfectum*** and ***F.oxysporum*** f. sp. ***melonis***. However these fungi were strongly mycoparasitic on ***Rhizoctonia solani*** and ***Pythium aphanidermatum***. When grown in liquid cultures containing either laminarin or chitin or fungal cell walls as sole carbon sources, two tested strains of ***T.harzianum*** excreted β-1,3-glucanase and chitinase into the medium. Higher levels of these enzymes were induced by hyphal cell walls of ***F.oxysporum*** in the mycoparasitic strain T-203 relative to that of T-35. Incubation of the lytic enzymes produced by T-35, with hyphal cell

walls of the test fungi released more glucose and N-acetyl-D-glucosamine from cell wall of ***R.solani*** and ***S.rolfsii*** than from those of ***F.oxysporum***. Treatments of cell wall (with 2N NaOH, protease or trypsin) prior to their incubation with the lytic enzymes of ***T.harzianum*** significantly increased the release of glucose and N-acetyl-D-glucosamine. These results suggest that proteins in cell walls of ***F.oxysporum*** may increase their resistance to degradation by extracellular enzymes from ***T.harzianum***. The significant biological control of ***F.oxysporum*** obtained by this strain (Sivan, 1989) may however be due to other mechanisms such as competition and/or induced resistance.

All the work reported above has involved the study of mycoparasitism by ***Trichoderma*** against plant pathogenic fungi. Little work has been down to date on the mycoparasitism of wood decay fungi. Hyphal interaction of ***T.harzianum*** and ***T.polysporum*** with wood decay fungi was studied by Murmanis ***et al.*** (1988 b). They studied the mycoparasitism of the above two fungi against wood decay fungi by observing: 1) hyphal interaction between these ***Trichoderma*** and wood decay fungi, using the SEM (Scanning electron microscopy); and 2) inhibition of decay fungi by filtrates of these ***Trichoderma*** and water extracts from wood permeated with these fungi. Water extracts from wood permeated with ***Trichoderma*** and culture filtrates did not inhibit the growth of the wood decay fungi on agar plates. Observations by SEM of the interactions of ***Trichoderma*** and the decay fungi most often showed their hyphae longitudinally attached to the hypahe of decay fungi. Hyphal coiling, hooks, and appressoria-like structures were also observed, but were infrequent. Spores of the ***Trichoderma*** were also found to be attached to the hyphae of decay fungi in a similar fashion to that of the parasitic hyphae. At the end of parasitic activity, ***T.harzianum*** and ***T.polysporum*** had totally consumed the hosts cytoplasmic contents, leaving behind only the "exoskeletons" of the hosts. The hyphae of decay fungi were void of cytoplasmic contents whether or not ***Trichoderma*** were present in the immediate vicinity.

Though the mycoparasitic effect of ***Trichoderma*** against wood decay fungi can be studied as described above, a good indication of the potential of an antagonist that affects decay fungi by parasitism can be determined on the basis of their production of

the two main lytic enzymes β- 1,3-glucanase and chitinase, as these are the key enzymes in the lysis of fungal cell walls (Mitchell and Alexander, 1963; Chet and Henis, 1969; Henis and Chet, 1975). The cell walls of wood decay fungi are composed of glucan and chitin (Jones ***et al.***, 1972) and as mentioned earlier, it is known that ***Trichoderma*** release lytic enzymes that can digest these components (Elad ***et al.***, 1983 a). These enzymes are reported to be constitutively produced by ***Trichoderma*** and by other fungi and yeasts that have glucan and chitin as part of their cell wall for the purpose of synthesis and autolysis during growth and also for the digestion of other plant material (Reese and Mandels, 1959; Bull and Chester, 1966). As with many other enzymes (eg., Cellulases of ***Trichoderma***) they can be induced and repressed by similar regulatory systems. Work done by Sivan and Chet (1986) has shown that the enzymes are inducible in the presence of cell wall material of plant pathogenic fungi and this might also be the case with wood decay fungal cell wall material.

One of the main aims of this section was therefore to study the inducibility of lytic enzymes by wood decay fungal cell wall material and to relate this to the importance of mycoparasitism in the antagonism of wood decay fungi by ***Trichoderma*** species.

3.3.2 Materials and Methods

The 10 selected ***Trichoderma*** isolates were inoculated (two cores of mycelium of 0.6 cm in diameter) into sterile liquid media (250 ml) of each of the two media types (MEB and LNM broth) and incubated in the dark for 14 days at either 22 or 25 °C. Fungal mycelium was removed by filtration and filtrates were sterilised by passing them through 0.45 µm sterile membrane filters (Whatman). The filtrates were then dialysed overnight (to remove excess sugars from the filtrates) in a continuously flowing cold water system at 10 to 12 °C using dialysis tubing of pore size 2.4 nm (Medicell Inter. Ltd.). Filtrates were then assayed for laminarinase and chitinase activity.

3.3.2.1 Laminarinase assay

Laminarinase catalysed depolymerisation of cell wall polymer, laminarin (Sigma L9634), was assayed by measuring the increases in reducing groups (glucose) at 40 °C using Nelson's modification of the Somogyi method (Nelson, 1944, as cited Green ***et al.***, 1989 a). The assays were done in special folin tubes (Baird and Tatlock, London, U.K.). Two folin tubes (X and Y) were used for each filtrate sample. One ml of a 10 mg/ml concentration of laminarin in 0.1 M citrate buffer at pH 5.0 (0.1 M sodium citrate and 0.1 M citric acid mixed at a volume ratio of 21:29 respectively) was added to each tube. One ml of the dialysed test filtrate was added to tube X which was incubated for 4 hrs in a 40 °C water bath covered with foil.

After the tubes were incubated 1 ml of the dialysed test filtrate was added to tube Y (Y was treated as a control). To both the tubes 2 ml of a copper reagent, consisting of 4 parts CuI reagent (16.1 g of $KNaC_4H_4O_6 \cdot 4H_2O$, 24 g of Na_2CO_3, 16 g of $NaHCO_3$, and 80.44 g of Na_2SO_4 was added to 800 ml of water, boiled until dissolved and the volume corrected to its original measure after cooling) and 1 part of CuII reagent (4 g of $CuSO_4 \cdot 5H_2O$, and 36 g of Na_2SO_4 was added to 200 mls of water, boiled until dissolved and the volume corrected to its original measure after cooling) was added to each tube. CuI and CuII were mixed together immediately before use. All the tubes were boiled for 10 min in a water bath at 100 °C to eliminate any enzymic activity and cooled. This was followed by addition of 2 ml of arsenomolybdate reagent

(25g of $(NH)_4Mo_7O_{24} . 4H_2O$ in 450 ml of H_2O + 21 ml of conc. H_2SO_4 + 3g of $Na_2HAsO_4 . 7H_2O$ dissolved in 25 ml of H_2O; the contents were mixed well and placed at 37 °C for 1-2 days before use) (all chemicals were obtained from BDH Mercks Ltd., Glasgow or Fisons, Glasgow, Scotland, unless otherwise referenced).

The volume in the individual tubes were made up to 25 ml by addition of distilled water. The samples were then mixed by vortexing and absorbance measured at 500 nm in a spectrophotometer. The blank used was a sample of water that went through the procedure from the incubation stage onwards i.e., had all the same reagents as the test samples. The final value of absorbance was used to extrapolate a reading for the amount of glucose released from the standard graph (see below) was the absorbance of X minus Y. Laminarinase activity of each test sample was obtained from an average of four replicates. A calibration curve was obtained by using varying concentrations of glucose ranging from 25, 50, 75, 100, 125, 150, 200, 250, 300, and 400 µg / 2 ml (Appendix 1) so that the amount of glucose released by enzymic action of each filtrate could be extrapolated (the standards were assayed using the same procedures as the test samples).

3.3.2.2 Chitinase Assay

For each test sample four boiling tubes were set up each containing 1 ml of dialysed filtrate and 3.8 mg of chitin (Sigma C3641) in 1 ml of citrate/phosphate buffer at pH 5.0 citrate/phosphate or McIlvaine buffer was prepared as follows. (Solution A - 0.1 M of citric acid and Solution B - 0.2 M of disodium phosphate mixed on the basis of the following formula, x ml A + (100-x) ml B where x = 49 ml). One tube from each set was then immediately boiled for 10 min to eliminate any enzyme activity (subsequently used as the control). All tubes were then incubated at 37 °C for 24 hrs.

After incubation the tubes were boiled at 100 °C for 10 min to stop enzyme activity. A 0.5 ml of the test sample, control or blank (the blank consisted of an equivalent volume of water going through the assay procedure) was added to 0.1 ml of 0.8 M potassium tetraborate, all the tubes were then heated in boiling water for 3 min and then cooled under tap water. Three ml of DMAB reagent was added and mixed in the tubes prior to incubation at 36 - 38 °C for 20 min (10g of DMAB or p-Dimethylaminobenzaldehyde

(Sigma D8904) dissolved in a solution mixture of 12.5 ml of 10 N HCl and 87.5 ml of Glacial acetic acid (Analar grade) and stored at 2 °C; the concentrated DMAB was diluted 1/10 in glacial acetic acid before use as a reagent. The samples were then cooled, vortexed to mix and absorbance read immediately at a wavelength 544 nm against a water blank treated by the same procedure. The average absorbance of the control tubes was subtracted from the average absorbance value of the test sample assays done in triplicate. A calibration curve was obtained by using varying concentrations of n-acetylglucosamine (Sigma A8625) ranging from 0, 1.6, 1.9, 3.1, 3.9, 6.25, 7.8, 12.5, 15.6, 25, 31.2, 40, 50, 62.5, 70, 80, 90, 100, 110, 120 and 125 µg / half ml obtained by serial dilutions of 1/2 or 1/10 (Appendix 1). Half ml quantities were used so that the amount of n-acetylglucosamine released by the enzymic activity of chitinase could be directly extrapolated from the standard graph (standards were processed using the same assay procedures as the test samples).

3.3.2.3 Induction of Laminarinase and Chitinase by Fungal Cell Walls

Induction of the two lytic enzymes laminarinase and chitinase in the ***Trichoderma*** isolates (the ten chosen isolates) were assayed by growing them in the low nutrient medium (LNM) autoclaved at 121 °C for 15 min, with addition of known amounts of cell wall preparations of seven different basidiomycetes. The treatment combinations used are listed as follows - 1) LNM; 2) LNM + cell wall material; 3) LNM - glucose + cell wall material. These filtrates were assayed using microassay systems as described in section 3.3.2.4.

3.3.2.3 (a) Basidiomycete cell wall preparation

The basidiomycetes (***N.lepideus*** FPRL 7F, ***T.versicolor*** MAD 697, ***G.trabeum*** MAD 617, ***P.Placenta*** MAD 698, ***A.carbonica*** HHB 5104, ***P.brevispora*** HHB 7030, ***I.lacteus*** HHB 7328) were grown in 4 litre volumes of sterile 3% (w/v) malt extract (Oxoid L39) broth at 25 °C on a shaker at a speed of 100 revolutions/min for a period of 14 days. After the period of incubation the mycelium was removed by filtering through Whatmann No.1 filter paper in a Buchner funnel. The mycelia were then frozen at - 20 °C. The methods that follow were adapted from Perez-Leblic ***et al.***, 1982 for preparation

of cell wall material. The mycelia were ground with a pestle and mortar then treated in an ultrasonic disintegrator (Braun-sonic 1510) for 3 min at 150 Watt. The ground mycelial pastes were then washed three times each by repeated centrifugation at 5000 - 6000 rpm first with 0.1 M NaCl and 0.5 M acetate buffer at pH 5.5 (0.5 M sodium acetate (anhydrous) and 0.5 M acetic acid mixed at a volume ratio of 6.2:1 respectively) followed by three washes with deionised distilled water. The cell wall materials were then lyophilised overnight and ground into a powder with a mortar and pestle.

3.3.2.3 (b) Culture of ***Trichoderma*** isolates on cell wall material

Based on work by Sivan and Chet (1989), 0.1g of cell wall material of each of the basidiomycetes were added to 50 ml volumes of liquid culture media (either LNM or LNM - glucose). The liquid media were then autoclaved at 121 °C for 15 min cooled and inoculated separately (a 0.6 cm diameter core of mycelium from the growing colony margin) with each of the appropriate 10 selected ***Trichoderma*** isolates. An additional set of flasks of LNM without cell walls were also set up and inoculated with the ***Trichoderma*** isolates. All possible combinations of ***Trichoderma*** isolates were thereby grown in each of the media with each of the seven basidiomycete cell wall materials. The inoculated liquid media were then incubated at 22 or 25 °C at 70 % humidity in the dark for a period of 14 days. After incubation the mycelium and residue cell wall material were removed by filtering through a 0.45 µm followed by a 0.22 µm sterile membrane filter (Whatman). The filtrates were stored at 4 °C for five days during which the laminarinase and chitinase assay were carried out.

Microassay for laminarinase and chitinase

The reagents used in both the enzyme assays were the same as described in sections 3.3.2.1 and 3.3.2.2. However a few differences in the assay procedure were as described below.

3.3.2.4 Laminarinase Microassay

The laminarinase microassay was conducted as described in the paper by Green ***et al.***, 1989 a. The microassay for reducing sugars was a modification of the Nelson-Somogyi assay (Nelson, 1944; Somogyi, 1952). In a 96-well microtitre plate (Dynatech,

Chantilly, U.S.A.) 25 µl of test sample and 25 µl of 10 mg/ml (or 1% w/v) of laminarin solubilised in 0.1 M citrate buffer at pH 5.0, were placed in each well. The plate was then covered with an acetate adhesive sheet and incubated at 40 °C for 24 hours. After incubation 75 µl of copper reagent as used before in the macroassay (section 3.3.2.1) was added, and the wells were resealed with the acetate sheet. The plate was then incubated at 80 °C for 30 min in a water bath. After the plate had cooled completely (15 min), 75 µl of arsenomolybdate (as used in the macroassay section 3.3.2.1) was added, and mixed on a vortex. Absorbance was measured at 500 nm with a Dynatech MR500 plate reader. A standard curve was prepared using glucose at concentrations ranging from 0, 250, 500 and 1000 µg/ml. The blank for reading both the standards and the test samples was a well containing all the reagents but with water replacing the filtrate.

3.3.2.5 Chitinase Microassay

The procedures for the laminarinase microassay were modified to suit the assay for chitinase, however all the reagents were the same as used in the macroassay. All the procedures up to and including incubation of test sample with chitin substrate overnight (approx 24 hours) at 37 °C were the same as described in section 3.3.2.2. After the incubation period tubes were boiled at 100 °C for 10 min to stop the enzymic reactions then 25 µl samples of the test sample, control, blank or standard were transferred into wells of a 96-well microtitre plate. To each 5 µl of 0.8 M potassium tetraborate was added. The plate was then covered with an acetate adhesive sheet and mixed on a vortex and then heated over steam for 3 min. After sufficiently cooling the plate 150 µl of DMAB (section 3.3.2.1) was added to the plates, resealed with the acetate sheet and then incubated at 36 - 38 °C for 20 min. The samples were then cooled and their absorbance at 544 nm measured on the Dynatech MR500 plate reader. A standard curve was prepared using n-acetylglucosamine at concentrations ranging from 0, 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 µg / half ml.

3.3.2.6 Microassays for detection of β-D-glucosaminidase and β-D-galactosaminidase

The two assays were adapted as a microassay from the original assay procedures reported in Highley, 1976. The substrates for the two enzymes were p-nitrophenyl-N-acetyl β-D-glucosaminide and p-nitrophenyl-N-acetyl β-D-galactosaminide (Sigma) for

the enzymes gluosaminidase and galactosaminidase respectively. Hundred µl of 0.05 % p-nitrophenyl substrate in 0.1 M acetate buffer (0.1 M of sodium acetate and 0.1 M acetic acid mixed in a volume ratio of 70:30 respectively) at pH 5.0 were mixed with 50 µl of culture filtrates and incubated at 40 °C for 1 hr. The reactions were terminated by the addition of 50 µl of 0.2 M $NaCO_3$. The resulting yellow colour was immediately measured for absorbance at 425 nm on the Dynatech MR500 plate reader. A unit of enzyme activity was defined as the amount required to liberate 0.001 µM of p-nitrophenol/hr. The standard curve was derived using p-nitrophenol of concentrations over the following range 0, 0.002, 0.003, 0.004, 0.005, 0.01, and 0.015 µM / 150 µl. The blank against which the test samples and standards were read was a mixture of substrate without the culture filtrate and buffer.

3.3.3 Results

3.3.3.1 Production of β-1,3 glucanase (laminarinase)

Table 3.3.3.1 (a) shows the levels of laminarinase produced by all the ***Trichoderma*** isolates. This enzyme (total activity) was produced in higher concentrations in the high nutrient malt medium, rather than in the low nutrient medium. There were however a few exceptions, B13 and B15, ***T.pseudokoningii*** spp. that were found to exhibit greater enzyme production in the low nutrient medium. These organisms may be of greater interest due to their ability to produce higher enzyme levels in a medium closer in nutritional consistency to wood. The ***Trichoderma*** isolates show interspecies variability, in the levels of laminarinase produced when considered as individual isolates eg., ***T.viride*** B5 produced 310 units of enzyme in the malt medium whereas ***T.pseudokoningii*** B13 produced only 60 units in the same medium. Similar interspecies variability was found in the low nutrient medium. Interstrain variability was also obvious, with ***T.viride*** R2 producing only 70 units of enzyme, which is less than a quarter of the amount produced by ***T.viride*** B5 (310 units) under the same conditions. This interstrain variability is also evident with the amount of enzyme produced in the low nutrient medium, however the variability within the individual isolates is comparatively less than that in the malt medium.

While total enzymic activity gives an indication of the level of enzyme produced, specific activity gives an indication of the level of enzyme produced relative to other proteins in the filtrate. Specific activity of laminarinase in the filtrates showed that, though the concentration of total protein produced in the malt medium is higher than that in the low nutrient medium, the relative proportion of laminarinase produced (i.e., specific activity) is greater in the low nutrient medium.

Mean values of the level of laminarinase total activity produced by all ***Trichoderma*** isolates in the malt and low nutrient medium was 178.63 and 120.82 units respectively (table 3.3.3.1 (b)). Analysis of variance (table 3.3.3.1 (c)) of the laminarinase total activity to examine the effect of media gave a significant value ($p<0.015$) indicating that laminarinase production is statistically different in the two media types. The mean laminarinase activity results (table 3.3.3.1 (b)) do not seem to indicate any obvious interspecies differences and is supported by the analysis of variance (table 3.3.3.1 (c)) testing where the effect of ***Trichoderma*** species gave an insignificant value ($p=0.249$) indicating that laminarinase production is not dependent on the ***Trichoderma*** species. It is evident that species subgroup 3 (***T.harzianum***) show the highest overall level of laminarinase production in the malt medium, however this is replaced by subgroup 4 (***T.pseudokoningii***) with highest enzyme level in the low nutrient media. This latter species are probably of more importance due to their higher enzyme production in the low nutrient medium. Though interspecies variability is shown not to be important in both media, the individual mean values of laminarinase activity in the LNM indicate greater interspecies variability than in the MEA. But it appears from standard deviations of the means in both media that the subgroups show some interstrain variability. Interstrain variability seems to be highest in species subgroup 2 (***T.viride***) in the MEA and in subgroup 4 (***T.pseudokoningii***) in the LNM.

Trichoderma Isolates	Malt media		Low Nutrient media	
	Total activity (units)	Specific activity (units)	Total activity (units)	Specific activity (units)
B1- T.aureoviride	151.25	2.61	75.62	30.24
B5- T.viride	310.83	5.33	57.58	25.03
R2- T.viride	70.77	1.16	72.5	21.32
R7- T.viride	206.76	0.38	47.52	37.41
R10- T.viride	173.45	3.46	90.89	72.71
B11- T.harzianum	255.32	4.44	238.67	136.38
B13- T.pseudokoningii	50.99	0.90	198.43	79.37
B14- T.pseudokoningii	190.1	3.30	151.25	121
B15- T.pseudokoningii	165.13	3.07	238.67	95.46
B16- Trichoderma unidentified	241.45	4.24	155.41	88.80

Table 3.3.3.1 (a) - Levels of production of laminarinase by ***Trichoderma*** isolates (Total activity represented as 1 unit = 1 µmole glucose released/ml of filtrate/hour. Specific activity represented as 1 unit = 1 µmole glucose released/hour/ug protein in filtrate).

Media type	Trichoderma species subgroups					
	1	2	3	4	5	All
Malt agar	150.5 (16.68)	191.2 (121.8)	256.7 (47.0)	123.4 (76.7)	243.5 (10.7)	178.63 (96.72)
Low nutr. media	75.2 (25.0)	67.1 (22.6)	120.7 (39.2)	196.1 (49.6)	155.4 (5.89)	120.82 (65.58)

Table 3.3.3.1 (b) - Mean total activity of laminarinase in both media types by the ***Trichoderma*** species subgroups. 1 - ***T.aureoviride*** ; 2 - ***T.viride*** ; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii*** ; 5 - unidentified ***Trichoderma.*** Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates of the species group tested. Standard deviations are in parentheses.

Factors	F ratio	Degree of freedom	P
Medium (M)	6.69	1, 30	p<0.015
Species subgp. (S)	1.43	4, 30	p=0.249
M x S	3.70	4, 30	p<0.015

Table 3.3.3.1 (c) - Analysis of variance of total laminarinase activity in the MEA and LNM (macroassay).

3.3.3.2 Production of chitinase enzyme

Table 3.3.3.2 (a) shows the levels of chitinase produced by the various ***Trichoderma*** isolates. Total chitinase activity is greater in the malt medium compared with the low nutrient medium with all isolates. Both interspecies and interstrain variability within the ***Trichoderma*** isolates was evident eg., ***T.harzianum*** B11 produced 41 units of chitinase in the malt medium whereas ***T.viride*** R10 produced only 0.07 units in the same medium; ***T.viride*** R2 however produced 25 units of chitinase. Similar to laminarinase the specific activity of chitinase is higher in the low nutrient medium, signifying that chitinase makes up a higher proportion of the total protein produced in the low nutrient

medium. There appears to be no direct relationship between chitinase and laminarinase production, however the overall level of chitinase produced in both media types is always much lower than that of laminarinase.

Mean values of the level of chitinase total activity produced by all ***Trichoderma*** isolates in the malt medium and the low nutrient medium was 8.65 and 1.03 respectively (table 3.3.3.2 (b)). This difference in enzyme production indicating the importance of media is supported by the analysis of variance testing. Table 3.3.3.2 (c) shows that the chitinase production is dependent on both the media type and the species subgroups, as indicated by their probability values (M, S and M x S = <0.001). Table 3.3.3.2 (b) shows the mean chitinase total activity by the ***Trichoderma*** species subgroups in the two media types, and species subgroup 3 (***T.harzianum***) shows the highest level of chitinase production in both the media types. It was noted that the same subgroup also produced the highest overall level of laminarinase in the malt medium. Species subgroup 4 (***T.pseudokoningii***) that showed highest levels of laminarinase in the LNM however does not produce equally high levels of chitinase in the same media. So there does not seem to be a correlation in the levels of chitinase and laminarinase production in the LNM which is of more interest due to it similarity in nutrient consistency to wood. Mean values of the total amount of chitinase produced by all species in the two media types (table 3.3.3.2 (b)) indicate that there is high interspecies variability, also proven by the analysis of variance testing. Individual standard deviations of the mean values of chitinase production for the species groups show that there is also interstrain variability within the species subgroups (with subgroup 2 (***T.viride***) showing the highest amount of interstrain variability).

Trichoderma Isolates	Malt media		Low nutrient media	
	Total activity (units)	Specific activity (units)	Total activity (units)	Specific activity (units)
B1- T.aureoviride	1.27	0.02	0	0
B5- T.viride	2.82	0.4 0·04?	2.35	1.02
R2- T.viride	25.2	0.41	0.56	0.16
R7- T.viride	0.94	0.017	0.28	0.22
R10- T.viride	0.07	0.001	0	0
B13- T.pseudokoningii	1.99	0.03	0.22	0.09
B14- T.pseudokoningii	0.47	0.008	0.09	0.072
B15- T.pseudokoningii	1.03	0.019	1.03	0.41
B11- T.harzianum	41.4	0.72	3.95	2.26
B16- Trichoderma unidentified	0.94	0.16	1.50	0.86

Table 3.3.3.2 (a) - Levels of production of chitinase by ***Trichoderma*** isolates (Total activity represented as 1 unit = 1 µmole n-acetylglucosamine released/ml of filtrate/hour. Specific activity represented as 1 unit = 1 µmole n-acetylglucosamine released/hour/ug protein in filtrate).

Media type	Trichoderma species subgroups					
	1	2	3	4	5	All
Malt agar	1.08 (0.60)	10.11 (10.2)	40.5 (2.93)	1.17 (0.71)	0.94 (0.53)	8.65 (13.3)
Low nutr. media	0 (0)	0.70 (0.97)	4.33 (0.53)	0.54 (0.52)	1.5 (0.13)	1.03 (1.35)

Table 3.3.3.2 (b) - Mean total activity of chitinase in both media types by the ***Trichoderma*** species subgroups. 1 - ***T.aureoviride*** ; 2 - ***T.viride*** ; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii*** ; 5 - unidentified ***Trichoderma***. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates of the species group tested. Standard deviations are in parentheses.

Factors	F ratio	Degree of freedom	P
Medium (M)	24.25	1, 30	p<0.001
Species subgp. (S)	15.51	4, 30	p<0.001
M x S	10.8	4, 30	p<0.001

Table 3.3.3.2 (c) - Analysis of variance of total chitinase activity in MEA and LNM (macroassay).

Production of laminarinase and chitinase (microassay)

3.3.3.3 Laminarinase and Chitinase activity in LNM (containing glucose)

Table 3.3.3.3 (a) gives the total activity of laminarinase and chitinase produced by the ***Trichoderma*** species in the low nutrient media, by the microassay. Comparisons of mean values of total laminarinase and chitinase activity in the ***Trichoderma*** species subgroups as assayed by both the macro and micro assay (Table 3.3.3.1 (b), 3.3.3.2 (b) and 3.3.3.3 (b)) in the low nutrient media showed that, subgroup 3 (***T.harzianum***) shows the highest level of laminarinase production by both assays. However, there seems to be more variability in the levels of chitinase produced by the two assay procedures. From the individual total enzyme activity of isolates and that of the species subgroups it is evident that, interspecies variability exist in the production of laminarinase more than that of chitinase. This is supported by the analysis of variance test to examine the effect of species on the production of (Table 3.3.3.3 (c)) enzymes which shows a significant value ($p<0.001$) only with laminarinase and not very significant with chitinase ($p=0.038$). Indicating that laminarinase production is statistically different within the species but this is however not very evident with chitinase. This could be due to the poorer reproducibility between replicates in the chitinase assays.

Trichoderma species	Laminarinase (total activity)	Chitinase (total activity)
B1- T.aureoviride	49.02	3.95
B5- T.viride	56.89	1.50
R2- T.viride	64.87	1.60
R7- T.viride	104.19	1.03
R10- T.viride	132.2	5.83
B11- T.harzianum	177.68	2.91
B13- T.pseudokoningii	195.06	6.78
B14- T.pseudokoningii	138.76	0.47
B15- T.pseudokoningii	137.98	1.92
B16- Trichoderma unidentified	136.62	3.95

Table 3.3.3.3 (a) - Levels of production of laminarinase and chitinase by ***Trichoderma*** isolates in the microassay in the low nutrient media (Total activity represented as 1 unit = 1 µmole of glucose or n-acetylglucosamine released/ml of filtrate/hour).

Media type	Trichoderma species subgroups				
	1	2	3	4	5
Low nutr. media (Laminarinase)	49.02 (0)	89.52 (30.74)	177.68 (0)	157.27 (27.0)	136.62 (0)
Low nutr. media (Chitinase)	3.95 (0)	2.49 (1.95)	2.91 (0)	3.05 (2.73)	3.95 (0)

Table 3.3.3.3 (b) - Mean total activity of laminarinase and chitinase in the low nutrient media (microassay) by the ***Trichoderma*** species subgroups. 1 - ***T.aureoviride*** ; 2 - ***T.viride*** ; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii*** ; 5 - unidentified ***Trichoderma***. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses.

Enzymes	Factors	F ratio	Degree of freedom	P
Laminarinase	Species subgps.	95.21	4, 135	p<0.001
Chitinase	Species subgps.	2.62	4, 135	p=0.038

Table 3.3.3.3 (c) - Analysis of variance of total laminarinase and chitinase activity in the low nutrient media.

3.3.3.4 Laminarinase and Chitinase activity in LNM (containing glucose) + basidiomycete cell wall

Table 3.3.3.4 (ai) and (aii) shows the total enzyme activity of laminarinase and chitinase respectively in the LNM containing seven different basidiomycete cell wall preparations.

From the study of the mean laminarinase activity of species subgroups with the seven basidiomycete cell walls (Table 3.3.3.4 (bi)) it was clear that there exists an enormous variation in the enzyme production within or between species, and these variations are also observed with presence of the different basidiomycete cell wall material. It was also evident from Table 3.3.3.4 (bii) that subgroup 1 (***T.aureoviride***) showed the highest level of laminarinase enzyme production although individual strains other species subgroups exhibited equally high enzyme production. Mean values of laminarinase activity (Table 3.3.3.4 (biii) in the presence of each of the seven basidiomycete cell walls show clearly that laminarinase production was very much dependent on the type of basidiomycete cell wall added in the medium. Mean laminarinase production by all the isolates was found to be considerably higher in LNM with cell wall material of brown rot fungi (27.3 total activity units) than white rot fungi (17 total activity units). So there is a certain degree of target specificity in the amount of enzyme produced. Laminarinase enzyme production seems to be highest in LNM with cell wall of ***N.lepideus*** among the brown rot fungi and with cell wall of ***T.versicolor*** among the white rot fungi. However, levels of enzyme produced with other basidiomycete cell wall material were also high. Analysis of variance undertaken to examine the effect of species and basidiomycete cell wall type (Table 3.3.3.4 (c)) showed that the cell wall type was more significant ($p<0.002$) in determining the level of enzyme production.

Trichoderma species	Laminarinase Total Activity						
	Basidiomycete cell walls						
	Brown rot fungi				White rot fungi		
	1	2	3	4	5	6	7
B1	41.1	48.5	21.5	34.6	28.8	16.9	0
B5	27.5	29.4	8.0	25.4	12.4	13.4	7.7
R2	39.3	1.5	6	46.8	2.8	12.3	40.3
R7	34.8	19	17.7	0	38.7	43.4	18.3
R10	45.6	33.9	8.9	62.5	14.6	13.9	43.2
B11	54.6	25.9	23.5	27.6	23.8	6.3	0
B13	14.7	27.6	24.9	30.5	16.6	8.2	18.9
B14	41.8	6.9	16.2	18.3	0	16.1	0
B15	37.8	29.2	12.6	28.9	39.8	10.5	0
B16	24	27.2	17.4	9.8	12.4	10.2	38.3

Table 3.3.3.4 (ai) - Levels of production of laminarinase by ***Trichoderma*** isolates in the low nutrient media containing basidiomycete cell wall; 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***. The cell wall of basidiomycetes 1 to 4 belong to brown rot fungi and 5 to 7 are white rot fungi. Amount of enzyme produced is represented as total activity, 1 unit of which is = 1 µmole of glucose released/ml of filtrate/hour.

Trichoderma species	Chitinase Total Activity						
	Basidiomycete cell walls						
	Brown rot fungi				White rot fungi		
	1	2	3	4	5	6	7
B1	0.84	10.9	38.0	3.01	0.09	4.70	3.67
B5	0.84	1.88	1.6	3.20	1.60	10.64	3.01
R2	1.41	1.41	0.37	1.60	3.95	13.18	0.84
R7	2.54	12.8	11.3	2.54	1.13	2.26	0.094
R10	0.37	4.7	1.6	8.94	0.094	1.41	0.094
B11	1.6	12.8	7.53	5.55	0.37	2.26	1.6
B13	0.09	7.72	0.75	8.47	0.094	1.13	12.99
B14	1.13	4.7	22.2	5.36	0.65	2.26	2.07
B15	0.65	1.13	2.26	12.05	1.13	19.21	2.26
B16	0.09	12.8	12.43	3.67	1.13	1.41	0.84

Table 3.3.3.4 (aii) - Levels of production of chitinase by ***Trichoderma*** isolates in the low nutrient media containing basidiomycete cell wall; 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***. The cell wall of basidiomycetes 1 to 4 belong to brown rot fungi and 5 to 7 are white rot fungi. Amount of enzyme produced is represented as total activity, 1 unit of which is = 1 µmole of n-acetylglucosamine released/ml of filtrate/hour.

The reason that the species effect was not very statistically significant is due to the variability in the level of enzyme produced by different strains of any one species. In a few cases the amount of enzyme produced was nil, this could be a catabolite repression effect seen due to the presence of glucose along with the cell wall material in the LNM, depressing the level of laminarinase production. It was also interesting to note that the amount of laminarinase produced in LNM without cell wall material shows higher levels than in LNM+cell wall material. This general reduction in the amount of laminarinase production could also be due to the catabolite repression effect due to excess available glucose.

Decay type	Trichoderma species subgroups				
	1	2	3	4	5
1	41.2 (0.2)	36.3 (7.07)	54.1 (0.6)	30.93 (13.09)	23.5 (0.75)
2	48 (0.33)	20.57 (13.1)	25.4 (0.73)	20.73 (11.1)	26.7 (0.70)
3	21 (0.2)	9.65 (4.82)	23 (0.65)	17.4 (5.6)	16.9 (0.68)
4	34.1 (0.25)	33.3 (24.9)	27.1 (0.63)	25.4 (5.95)	9.3 (0.65)
5	28.3 (0.23)	16.6 (14.14)	23.3 (0.7)	41.8 (58.4)	11.9 (0.73)
6	16.4 (0.2)	20.2 (14.0)	5.8 (0.73)	11.1 (3.6)	9.7 (0.64)
7	0 (0)	26.8 (15.9)	0 (0)	6.1 (9.5)	37.8 (0.76)

Table 3.3.3.4 (bi) - Mean total activity of laminarinase in the five ***Trichoderma*** species subgroups in the low nutrient media containing seven different basidiomycete cell wall material. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses. Basidiomycete cell walls: 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. ***Trichoderma*** species: 1 - ***T.aureoviride***; 2 - ***T.viride***; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii***; 5 - unidentified ***Trichoderma***.

Trichoderma species subgroups (As per table 3.3.3.4 (bi))				
1	2	3	4	5
27.0 (15.5)	23.3 (16.4)	22.6 (16.7)	21.9 (24.5)	19.4 (10.1)

Table 3.3.3.4 (bii) - Mean total activity of laminarinase in the five ***Trichoderma*** species subgroups in the low nutrient media+glucose and basidiomycete cell wall material (irrespective of the type of cell wall material). Each value in the table is the mean for all replicates of ***Trichoderma***

Basidiomycete cell wall type						
1	2	3	4	5	6	7
37.2	28.28	17.59	25.84	24.3	12.65	14.16

Table 3.3.3.4 (biii) - Mean total activity of laminarinase in the low nutrient media containing basidiomycete cell wall material, irrespective of the ***Trichoderma*** species subgroups. Each value in the table is the mean of laminarinase total activity by all the ***Trichoderma*** isolates against each type of basidiomycete cell wall material. 1 to 4 are brown rot fungal cell wall material and 5 to 7 is that of white rot fungi.

Factors	F ratio	Degree of freedom	P
Species subgps.(S)	0.40	4, 105	p=0.808
Decay cell wall(D)	3.69	6, 105	p<0.002
D X S	1.49	24, 105	p=0.088

Table 3.3.3.4 (c) - Analysis of variance of total laminarinase activity in the low nutrient media+glucose and basidiomycete cell wall material (medium 2).

In the case of production of the chitinase enzyme, again there was very high variability in the amount of enzyme produced (Table 3.3.3.4 (di)). Mean values of chitinase activity within each species subgroups irrespective of the type of basidiomycete cell wall material (table 3.3.3.4 (dii) shows that subgroup 1 (***T.aureoviride***) produces the highest amount of chitinase.

Decay type	Trichoderma species subgroups				
	1	2	3	4	5
1	0.79 (0.71)	1.15 (0.72)	1.6 (0)	0.62 (0.46)	0.09 (0)
2	10.4 (0.7)	4.94 (4.69)	12.3 (0.7)	4.18 (2.76)	12.3 (0.7)
3	37.5 (0.7)	3.59 (4.48)	7.03 (0.7)	8.23 (10.45)	11.9 (0.7)
4	3.01 (0)	3.69 (2.99)	5.05 (0.7)	8.06 (3.04)	3.1 (0.7)
5	0.09 (0)	1.56 (1.32)	0.37 (0)	0.62 (0.46)	1.13 (0)
6	4.2 (0.7)	6.46 (5.32)	1.76 (0.7)	7.17 (8.94)	1.41 (0)
7	3.17 (0.7)	0.882 (1.08)	1.6 (0)	5.41 (5.43)	0.84 (0)

Table 3.3.3.4 (di) - Mean total activity of chitinase in the five ***Trichoderma*** species subgroups in the low nutrient media containing seven different basidiomycete cell wall material. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses. Basidiomycete cell walls, 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, 1 - ***T.aureoviride***; 2 - ***T.viride***; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii***; 5 - unidentified ***Trichoderma***.

Mean values of chitinase activity (table 3.3.3.4 (diii) in the presence of each of the seven basidiomycete cell walls showed that chitinase production is dependent on the type of cell wall present in medium. Mean chitinase production by all the isolates was found to be higher overall in the presence of brown rot fungal cell wall (6.97 total activity units) than with white rot fungal cell wall (2.4 total activity units). So as with laminarinase again there is a certain degree of target specificity in the amount of enzyme produced. And chitinase levels seem to be highest in the presence of the brown rot fungal cell wall of ***P.placenta*** and cell wall of ***P.brevispora*** among the white rot fungi. Analysis of

variance undertaken to examine the effect of species and basidiomycete cell wall type (table 3.3.3.4 (e)) showed that both the factors play a significant role in determining the level of enzyme produced. It is of some interest that the overall levels of chitinase produced in LNM containing cell walls is higher than in LNM alone unlike laminarinase, and indicates enzyme induction in the presence of the basidiomycete cell wall material.

Trichoderma species subgroups (As per table 3.3.3.4 (di))				
1	2	3	4	5
8.45 (12.7)	3.18 (3.7)	4.24 (4.1)	4.90 (6.1)	4.39 (5.1)

Table 3.3.3.4 (dii) - Mean total activity of chitinase in the five ***Trichoderma*** species subgroups in the low nutrient media containing basidiomycete cell wall materials (irrespective of the type of cell wall material). Each value in the table is the mean for all replicates of ***Trichoderma***

Basidiomycete cell wall type (As per table 3.3.3.4 (di))						
1	2	3	4	5	6	7
0.85	8.82	13.65	4.58	0.75	4.20	2.38

Table 3.3.3.4 (diii) - Mean total activity of chitinase in the low nutrient media containing basidiomycete cell wall material, irrespective of the ***Trichoderma*** species subgroups. Each value in the table is the mean of laminarinase total activity by all the ***Trichoderma*** isolates against each type of basidiomycete cell wall material. 1 to 4 are brown rot fungal cell wall material and 5 to 7 are that of white rot fungi.

Factors	F ratio	Degree of freedom	P
Species subgps.(S)	4.8	4, 105	p<0.001
Decay cell wall(D)	18.29	6, 105	p<0.001
D X S	5.04	24, 105	p<0.001

Table 3.3.3.4 (e) - Analysis of variance of total chitinase activity in the low nutrient media containing basidiomycete cell wall material.

3.3.3.5 Laminarinase and chitinase activity in LNM-glucose+basidiomycete cell wall

Table 3.3.3.5 (ai and aii) shows the total enzyme activity of laminarinase and chitinase respectively in the LNM with seven different basidiomycetes cell wall preparations.

The very high levels of laminarinase production in this medium in comparison to that in LNM containing glucose and cell wall material, indicates that the laminarinase activity was highly repressed in the presence of glucose in the latter medium. From the study of the mean laminarinase activity of species subgroups with the seven basidiomycete cell walls (table 3.3.3.5 (bi)) it was evident that the variation within the species subgroups and cell wall types is not as obvious as seen in LNM containing both glucose and cell wall (table 3.3.3.4 (bi)). Mean values of laminarinase production in the species subgroups irrespective of cell wall material (table 3.3.3.5 (bii)) showed that subgroup 4 (***T.pseudokoningii***) produced the highest level of laminarinase enzyme, unlike in the same medium containing glucose where it was subgroup 1, (***T.aureoviride***). Mean values of laminarinase activity in the presence of each of the seven basidiomycete cell walls showed that laminarinase production is dependent on the cell wall type (table 3.3.3.5 (biii)). Mean laminarinase production by all isolates was not found to be that different in the presence of brown rot fungal cell wall (114.2 total activity units) and white rot fungal cell wall (111.3 total activity units), as seen in LNM with glucose and cell wall. However this could be a masked effect seen due to the exceptionally high levels of laminarinase produced in the presence of the white rot cell wall of ***T.versicolor***, while the other two cell wall types cause a lower level of enzyme to be produced in their presence.

Trichoderma species	Laminarinase Total Activity Basidiomycete cell walls						
	Brown rot fungi				White rot fungi		
	1	2	3	4	5	6	7
B1	103.4	84.3	77.0	76.7	91.4	93.4	96.84
B5	127.5	121.5	85.5	90.9	118.9	89.5	94.6
R2	126.3	101.3	80.4	78.8	138.9	107.5	84.2
R7	102.1	81.5	78.9	82.7	91.3	95.2	78.5
R10	174.6	115.5	79.9	123.5	205.0	85.7	103.5
B11	84.6	126.0	96.8	77.0	77.7	76.8	82.8
B13	216	108.6	171.7	80.3	196.9	77.6	95.7
B14	142.1	118.5	119.6	79.4	126.2	90.5	87.7
B15	316.1	257.2	259.7	133.9	362.3	91.8	100.5
B16	179.9	128.4	96.2	85.2	182.4	123.9	123.2

Table 3.3.3.5 (ai) - Levels of production of laminarinase by ***Trichoderma*** isolates in the low nutrient media minus glucose and with basidiomycete cell wall (medium 3); 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***Ph.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***. The cell wall of basidiomycetes 1 to 4 belong to brown rot fungi and 5 to 7 are white rot fungi. Amount of enzyme produced is represented as total activity, 1 unit of which is = 1 µmole of glucose released/ml of filtrate/hour.

Trichoderma species	Chitinase Total Activity Basidiomycete cell walls						
	Brown rot fungi				White rot fungi		
	1	2	3	4	5	6	7
B1	17.51	10.64	8.0	0.09	4.89	9.22	5.36
B5	26.74	9.41	9.13	3.01	32.39	9.88	6.96
R2	2.26	3.67	18.83	3.67	0.09	17.70	5.65
R7	6.59	22.2	10.36	0.18	45.01	4.14	7.724
R10	5.83	24.67	8.47	3.95	4.70	1.13	2.26
B11	2.26	14.31	19.96	0.09	1.6	11.67	2.26
B13	18.27	3.48	7.25	1.88	4.7	6.02	6.02
B14	0.37	1.31	6.02	2.73	2.63	7.53	1.60
B15	6.96	8.19	4.70	1.88	44.83	8.66	2.26
B16	2.07	10.17	23.35	7.25	6.78	7.72	5.65

Table 3.3.3.5 (aii) - Levels of production of chitinase by ***Trichoderma*** isolates in the low nutrient media minus glucose and with basidiomycete cell wall (medium 3); 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***Ph.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***. The cell wall of basidiomycetes 1 to 4 belong to brown rot fungi and 5 to 7 are white rot fungi. Amount of enzyme produced is represented as total activity, 1 unit of which is = 1 µmole of n-acetylglucosamine released/ml of filtrate/hour.

Laminarinase production seems to be highest with cell wall type of ***N.lepideus*** among the brown rot fungi and with cell wall of ***T.versicolor*** among the white rot fungi. These are the same cell wall types that also showed a high level of laminarinase in the LNM containing glucose and cell wall material. Analysis of variance tests undertaken to examine the effect of species and basidiomycete cell wall type (table 3.3.3.5 (c)) showed that both the factors have a significant role to play in the level of enzyme produced. It was also noted that the level of enzyme produced in all cases was far higher, than in either LNM or LNM with glucose and cell wall material. The results produced in the absence of glucose in the LNM+cell wall material show the level of enzyme produced unrepressed compared with the repressed levels seen in the other two media.

Decay type	**Trichoderma species subgroups** (as in table 3.3.3.5 (aii))				
	1	2	3	4	5
1	102.9 (0.83)	132.1 (28.09)	84.1 (0.71)	224.2 (78.1)	179.4 (0.90)
2	83.8 (0.7)	104.4 (16.4)	125.5 (0.64)	160.9 (74.3)	127.9 (0.76)
3	76.5 (0.71)	80.68 (2.78)	96.3 (0.65)	183.1 (63.34)	95.7 (0.75)
4	76.2 (0.82)	93.47 (18.82)	76.5 (0.76)	97.3 (27.92)	84.7 (0.83)
5	90.9 (0.77)	138 (44.83)	77.2 (0.68)	227.9 (108.38)	181.9 (0.88)
6	92.9 (0.74)	93.97 (8.83)	76.3 (0.77)	86.13 (7.04)	123.4 (0.70)
7	96.34 (0.75)	89.7 (10.2)	82.3 (0.76)	94.13 (5.81)	122.7 (0.86)

Table 3.3.3.5 (bi) - Mean total activity of laminarinase in the five ***Trichoderma*** species subgroups in the low nutrient media minus glucose with seven different basidiomycete cell wall material. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses. Basidiomycete cell walls, 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***.

Trichoderma species subgroups (As per table 3.3.3.5 (bi))				
1	2	3	4	5
88.51 (9.71)	104.63 (29.71)	88.31 (17.14)	153.42 (82.26)	130.81 (36.08)

Table 3.3.3.5 (bii) - Mean total activity of laminarinase in the five ***Trichoderma*** species subgroups in the low nutrient media minus glucose and with basidiomycete cell wall materials (irrespective of the type of cell wall material). Each value in the table is the mean for all replicates of ***Trichoderma***

Basidiomycete cell wall type (As per table 3.3.3.5 (bi))						
1	2	3	4	5	6	7
144.5	120.5	106.47	85.65	143.2	94.54	97.03

Table 3.3.3.5 (biii) - Mean total activity of laminarinase in the low nutrient media minus glucose and with basidiomycete cell wall material irrespective of the ***Trichoderma*** species subgroups. Each value in the table is the mean of laminarinase total activity by all the ***Trichoderma*** isolates against each type of basidiomycete cell wall material. 1 to 4 are brown rot fungal cell wall material and 5 to 7 is that of white rot fungi.

Factors	**F ratio**	**Degree of freedom**	**P**
Species subgps.(S)	14.1	4, 105	$p<0.001$
Decay cell wall(D)	4.91	6, 105	$p<0.001$
D X S	2.05	24, 105	$p<0.007$

Table 3.3.3.5 (c) - Analysis of variance of total laminarinase activity in the low nutrient media minus glucose and with basidiomycete cell wall material.

In the case of production of the chitinase enzyme, again there was very high variability in the amount of enzyme produced (table 3.3.3.5 (di)). Mean values of chitinase activity within each species subgroup irrespective of the type of basidiomycete cell wall material (table 3.3.3.5 (dii)) show that subgroup 2, (***T.viride***) produces slightly higher amounts of chitinase although the other subgroups produce similar levels of enzyme. Analysis of variance (table 3.3.3.5 (e)) carried out to examine the effect of species subgroups and

cell wall type on the level of enzyme production show that basidiomycete cell wall type has a more significant role to play ($p<0.016$), than the species subgroups ($p=0.27$). Mean chitinase production by all the isolates was not found to be very different between LNM with the brown rot (8.12 total activity units) and white rot cell wall material (7.42 total activity units) (table 3.3.3.5 (diii). This pattern was similar to that found with the production of laminarinase.

Decay type	Trichoderma species subgroups				
	1	2	3	4	5
1	17.0 (0.68)	9.98 (10.1)	1.7 (0.50)	8.15 (7.8)	1.5 (0.7)
2	10.1 (0.56)	14.4 (9.3)	8.8 (7.7)	3.9 (2.9)	9.6 (0.78)
3	7.5 (0.64)	11.1 (4.5)	19.4 (0.7)	5.46 (1.24)	22.8 (0.77)
4	0.09 (0)	2.31 (1.43)	0.09 (0)	1.93 (0.37)	6.7 (0.71)
5	4.3 (0.53)	20.14 (19.9)	1.6 (0)	17.0 (21.1)	6.2 (0.68)
6	8.7 (0.66)	7.8 (6.63)	11.1 (0.7)	6.86 (1.28)	7.2 (0.65)
7	4.8 (0.69)	5.22 (2.08)	2.2 (0)	3.1 (1.9)	5.1 (0.69)

Table 3.3.3.5 (di) - Mean total activity of chitinase in the five ***Trichoderma*** species subgroups in the low nutrient media minus glucose and with the seven different basidiomycete cell wall material. Each value in the table is the mean for all replicates of all ***Trichoderma*** isolates tested. Standard deviations are in parentheses. Basidiomycete cell walls: 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species: 1 - ***T.aureoviride***; 2 - ***T.viride***; 3 - ***T.harzianum***; 4 - ***T.pseudokoningii***; 5 - unidentified ***Trichoderma***.

Trichoderma species subgroups (As per table 3.3.3.5 (di))				
1	2	3	4	5
7.49 (5.15)	10.14 (10.6)	6.41 (7.13)	6.64 (9.3)	8.44 (6.5)

Table 3.3.3.4 (dii) - Mean total activity of chitinase in the five ***Trichoderma*** species subgroups in the low nutrient media minus glucose and with basidiomycete cell wall materials (irrespective of the type of cell wall material). Each value in the table is the mean for all replicates of ***Trichoderma***

Basidiomycete cell wall type (As per table 3.3.3.5 (di))						
1	2	3	4	5	6	7
7.65	9.37	13.26	2.22	9.85	8.33	4.08

Table 3.3.3.5 (diii) - Mean total activity of chitinase in the low nutrient media minus glucose and with basidiomycete cell wall material, irrespective of the ***Trichoderma*** species subgroups. Each value in the table is the mean of laminarinase total activity by all the ***Trichoderma*** isolates against each type of basidiomycete cell wall material. 1 to 4 are brown rot fungal cell wall material and 5 to 7 are that of white rot fungi.

Factors	**F ratio**	**Degree of freedom**	**P**
Species subgps.(S)	1.31	4, 105	p=0.270
Decay cell wall(D)	2.74	6, 105	p<0.016
D X S	1.25	24, 105	p=0.220

Table 3.3.3.5 (e) - Analysis of variance of total chitinase activity in the low nutrient media minus glucose and with basidiomycete cell wall material.

However this could be due to the very high variability in the amount of chitinase produced in the presence of different cell wall types. Chitinase production seems to be highest with cell wall type 3 (***P.placenta***) among the brown rots and with cell wall type 5 (***T.versicolor***) among the white rots. It is evident from Figure 3.3.3 (a) that laminarinase production is catabolite repressed in the LNM media with glucose and cell wall. However chitinase production (Figure 3.3.3 (b)) does not seem to be as repressed

as laminarinase in the presence of glucose. Interspecies and interstrain variability among the isolates is also evident from the two graphs. The levels of enzyme produced in LNM with cell wall but no glucose represents the unrepressed level of enzyme activity and therefore can be considered of more importance than the other two media. The results indicate that ***T.pseudokoningii*** spp. show the highest amounts of laminarinase activity and ***T.viride*** show high levels of chitinase production. There does not therefore seem to be any correlation in the production of the two enzymes. However it is very clear from the results reported earlier that the levels of both enzymes produced is dependent on the type of basidiomycete cell wall material in the medium.

3.3.3.6 Glucosaminidase and galactosaminidase activity in LNM, LNM + cell wall material and LNM - glucose + cell wall material

Neither of the enzymes could be detected in LNM and LNM + cell wall material. However levels of activity of both enzymes were detected in LNM - glucose + cell wall material. ***Trichoderma*** isolates B11 (***T.harzianum***), B13 and B15 (***T.pseudokoningii***) produce higher levels of both enzymes with most cell wall types. The latter species also exhibit high levels of laminarinase, but not of chitinase. Induction of the two enzymes appears to be higher in the presence of cell wall type 2 (***G.trabeum***) but this has no correlation to any induction seen with laminarinase or chitinase. In general it is evident that higher levels of glucosaminidase production is matched by equally higher levels of galactosaminidase.

Figure 3.3.3 (a) - Laminarinase (total activity) produced by the ***Trichoderma*** species subgroups in LNM, LNM+G+CW and LNM-G+CW.

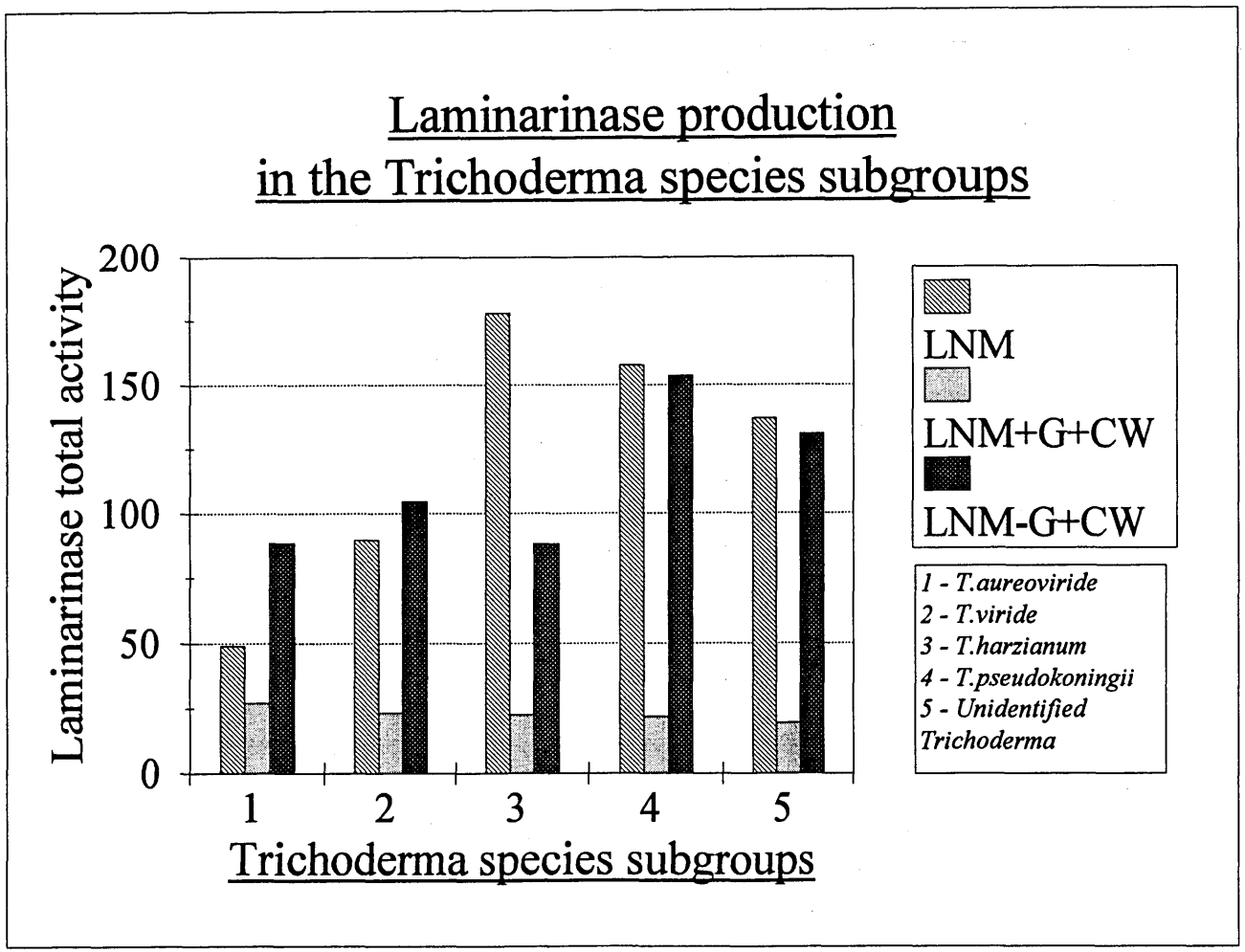
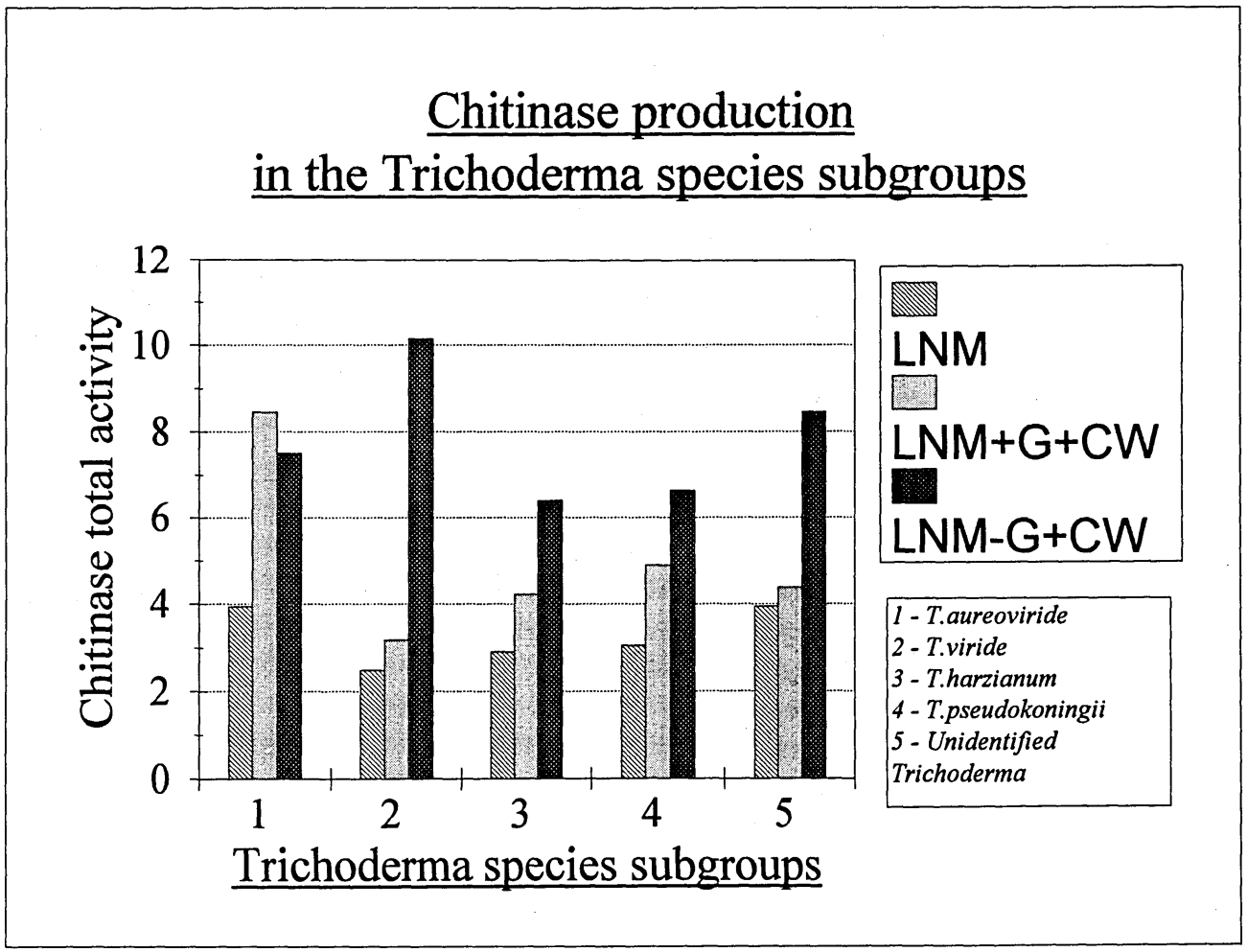


Figure 3.3.3 (b) - Chitinase (total activity) produced by the ***Trichoderma*** species subgroups in LNM, LNM+G+CW and LNM-G+CW.



Trichoderma species	Basidiomycetes cell walls													
	1		2		3		4		5		6		7	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
B1	1	1	4	2	4	2	2	2	1	2	2	2	2	1
B5	1	1	4	2	2	2	2	2	1	1	2	4	2	1
R2	2	1	4	4	2	2	1	1	1	1	2	2	2	2
R7	1	1	8	2	2	2	2	2	1	1	2	2	2	1
R10	4	2	2	2	2	2	2	1	1	1	2	2	2	1
B11	2	2	4	4	2	2	4	6	2	2	2	2	2	2
B13	2	2	8	8	2	2	4	6	2	2	2	4	4	8
B14	1	1	2	2	2	2	2	2	2	2	2	4	2	2
B15	1	1	4	4	4	6	2	2	2	2	2	10	2	2
B16	1	1	4	2	2	2	2	1	1	1	4	4	2	2

Table 3.3.3.6 - Levels of production of glucosaminidase (A) and galactosaminidase (B) by ***Trichoderma*** isolates in the low nutrient media minus glucose containing basidiomycete cell wall; 1 - ***N.lepideus***, 2 - ***G.trabeum***, 3 - ***P.placenta***, 4 - ***A.carbonica***, 5 - ***T.versicolor***, 6 - ***P.brevispora***, 7 - ***I.lacteus***. The ***Trichoderma*** species, B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - unidentified ***Trichoderma***. The cell wall of basidiomycetes 1 to 4 belong to brown rot fungi and 5 to 7 are white rot fungi. Amount of enzyme produced is represented as follows - 1 unit of enzyme activity is equivalent of 0.001 µM of p-nitrophenol released/hr.

3.3.4 Discussion

As reviewed in the introduction, it has been known that laminarinase and chitinase are key enzymes involved in the lysis of fungal cell walls, mainly from the work done with plant pathogenic fungal cell wall material. It has also been noted by the same authors that the enzymes are inducible in the presence of the plant pathogenic fungal cell wall material and the results presented above indicate that laminarinase and chitinase are also induced by the presence of cell wall material from wood decay basidiomycetes.

The ten ***Trichoderma*** isolates selected previously on the basis of interaction studies (Chapter 2) were all tested for the levels of lytic enzyme production in MEA (malt extract agar) and LNM (low nutrient medium). Levels of the two enzymes laminarinase and chitinase tested by macroassay (sections 3.3.3.1 and 3.3.3.2) showed that their production was far higher in MEA than LNM (Srinivasan ***et al.***, 1992 a, b). It is obvious from the results that the production of enzyme is influenced by the nutrient consistency of the medium. However, only the levels of enzyme produced in the LNM can be considered to be near the realistic amounts produced ***in vitro*** as the LNM is devised to have a C:N ratio similar to that of wood.

The influence of various nitrogen sources on the production of cellulase enzyme by several strains of ***T.harzianum*** has been studied by Dwivedi and Sharma (1989). It is evident from their work that organic nitrogen nutrients induce greater enzyme production than inorganic nitrogen. This correlates with the results obtained in this study where levels of the lytic enzymes were greater in MEA than LNM, the former being richer in organic nitrogen nutrients. However, there were a few exceptions among the isolates that produced equally high levels of enzyme in the LNM, and these are likely to be of greater interest for further study due to their ability to produce higher enzyme levels in a medium closer in nutrient consistency to wood. It is important to bear in mind however that these levels of enzyme are only constitutive levels produced by the antagonist in the presence of glucose as its sole source of carbon. Work carried out by Acebal ***et al.*** (1988) on the influence of different carbon sources on the production of endoglucanase and β-glucosidase in ***T.reesei*** showed that wheat straw as

the sole carbon source gave the highest level of enzyme production in comparison to xylan and cellulose. They also noted that β-glucosidase activity varied with time of culture and reached its highest level after 14 days of growth, which was the time of growth that was allowed for all the ***Trichoderma*** cultures in this study before assaying for enzymes.

Whipps and Magan (1987) have pointed out the importance of using low nutrient media when studying interactions between antagonists and plant pathogens because these fungi complete their life cycles under nutrient-poor conditions. Tests carried out on high nutrient media might not correspond with natural conditions. This is also likely to be true for mycoparasitic relationships. The work of Persson and Baath (1992) has shown that the mycoparasitism of ***Rhizoctonia solani*** by ***Arthrobotrys oligospora*** was influenced by nutrient levels. The type of carbon and nitrogen concentration in the medium was found to influence the coiling frequency of the mycoparasite which could be quantified. Their hypothesis was that coiling frequency would increase at low nutrient concentrations, since nutrients gained from parasitising other fungi could be expected to be more important under such conditions. Instead they found that increasing concentration of corn meal agar gave increasing coiling frequency up to a concentration of half the recommended strength. At higher concentrations the coiling frequency was constant, although the hyphal density of both fungi increased over the whole concentration range. Coiling frequency was positively correlated with probability of hyphal encounter, calculated as the product of the hyphal densities of the two fungi, except at high corn meal agar concentrations. Therefore, although hyphal densities were related to the nutrient level, the increased coiling frequency could be explained by the higher probability of encounter between hyphae of the two species at higher nutrient levels. The findings of Persson and Baath (1992) suggests that mycoparasitic fungi can change their growth pattern according to level of the nutrients and so it may be reasonable to hypothesise that mycoparasitism by ***Trichoderma*** may be also dependent on nutrient availability. The results of the study here clearly show the the nutrient content of the medium is a determinant of the levels of lytic enzyme produced. So it appears that the physical and chemical responses of a fungi are interrelated to the nutrient composition of the medium of growth.

Amongst several carbohydrates tested by Persson and Baath (1992) glucose resulted in the highest, and sucrose the lowest, coiling frequency. The effect of the different carbohydrates on coiling frequency was not correlated with hyphal densities of the fungi. Addition of a nitrogen source, $NaNO_3$, removed the differences in coiling frequency between glucose and sucrose and increased coiling frequencies on both sugars.

These authors however have not considered the possibility that higher nutrient levels or different carbohydrate sources apart from increasing the hyphal density may also alter the physiological aspects of the antagonist and pathogen. It is possible that the stage preceded by coiling, i.e., the recognition stage which may involve the role of lectins as discussed in the introduction may also have a role to play in determining the degree of coiling. It is observed by Elad ***et al.*** (1983 a) that a lectin present in ***Rhizoctonia solani***, binds to galactose residues on ***Trichoderma*** cell walls. Similarly a more specific recognition between ***S.rolfsii*** and ***Trichoderma*** was also found to be related to lectins, however the binding site of this agglutinin on ***Trichoderma*** was D-glucose and D-mannose provided the cations Mn^{2+} and Ca^{2+} were present (Barak ***et al.***, 1985). Such agglutinins had been purified earlier in the work by Mirelman ***et al.*** (1975) on a sepharose column and its separation on polyacrylamide gels showed two bands indicating two subunits which was subsequently found to be a glycoprotein with lectin characteristics.

It is clear from this that both the specific lectins (glycoprotein) produced by pathogens and the attachment sites on the antagonists are likely to vary dependent on the growth media (carbon and nitrogen source), thereby influencing the coiling frequency, as this will be related to the number of sites of hyphal interaction. Therefore it is important to chose an appropriate media for any mycoparasitic studies as well as for assay of lytic enzymes that are involved later in the interaction process. The LNM used here only gives the constitutive level of enzyme that can be produced by ***Trichoderma*** and the results of enzyme levels produced in LNM with basidiomycete cell wall material should give a better indication of the levels of enzyme production in wood. The enzyme levels

in MEA are obviously exaggerated due to high nutrient levels and may not represent mycoparasitic potential of the antagonists. The importance of selecting the right media was also supported by the analysis of variance testing that showed that media type has a significant role to play in determining the levels of production of both laminarinase and chitinase.

Even the levels of enzyme produced in the presence of the crude cell wall preparations, however may not truely represent the levels of enzyme produced in the actual encounter of the antagonist and decay fungi ***in situ***, as the cellular components at the antagonistic binding site may not be complete on the cell wall material. However, due to the variations that have been observed with the level of enzyme production with different basidiomycete cell wall material, and because the cell walls were not treated with any proteases during extraction, it is possible that there is some trace of glycoprotein material that the antagonists can recognise, which may explain the variation in the levels of enzyme production.

Both the production of laminarinase and chitinase in the LNM as measured by the microassay showed similar levels of enzyme activity as seen with the macroassay. Study of the results obtained in LNM and analysis of variance of the enzyme levels in this media (table 3.3.3.3 (c)) show that, the production of laminarinase and chitinase varies depending on the ***Trichoderma*** species. However, it is of more interest to analyse the enzyme results obtained in LNM with cell wall material. It was found that in LNM containing glucose and cell wall material the amount of laminarinase produced was lower than seen in the LNM alone, however the chitinase production was slightly higher. It is evident from the results that in the presence of both the cell wall material and glucose laminarinase production by ***Trichoderma*** spp. remains repressed due to catabolite repression by the glucose. Analysis of variance testing showed that both enzymes were dependent on the type of cell wall material added in the media, but only the levels of chitinase enzyme showed a correlation which was dependent on the ***Trichoderma*** species tested.

Considering only the results of laminarinase in the three media, it would appear that the levels of the enzyme were repressed due to the presence of glucose in the media (end product inhibition or catabolite repression) as the breakdown product of laminarinase in the cell wall is glucose and excess glucose in the media will affect the production of the enzyme. Such catabolite repression effects have been observed by Tiunova ***et al.*** (1983) with both laminarinase and chitinase with ***T.viride***. It is also known that cellulases of ***T.reesei*** are subject to a number of biochemical and genetic controls and one of the most important of these is end product inhibition. The results presented here show such catabolite repression of laminarinase in the LNM + cell wall material with the glucose compared to LNM with only cell wall material and no glucose. The complete repression by glucose of laminarinase production in some isolates accounts for the insignificant variability between species subgroups recorded in the LNM containing cell walls (table 3.3.3.4 (c)).

Chitinase production unlike that of laminarinase shows less repression in the presence of glucose. The reason for a lesser inhibitory effect of glucose could be attributed to the fact that the breakdown product of chitin is n-acetylglucosamine, and therefore there will be no end product inhibition involved. However the levels of chitinase produced in the medium containing cell walls but no glucose are higher than that produced when glucose and cell walls are both present, because in the latter media glucose is available as a readily utilisable carbon source and therefore the organisms spend less energy in utilising the cell wall material. Such effects of repression and induction have been studied by Ulhoa and Peberdy (1991) with chitinase synthesis in ***T.harzianum***. Chitinase was found to be induced on chitin-containing media, but repressed by glucose and n-acetylglucosamine.

The results clearly show that the addition of the basidiomycete cell wall material to the media is very important in determining the level of enzyme production. Though the levels of production of both enzymes seem to be greater in the presence of the brown rot cell wall material than that of white rot. This effect was not as evident when the glucose was removed from the medium. It has been discussed in the earlier chapters that "hyphal sheaths" of basidiomycetes may play a protective role in these fungi. The target

specificity against brown rot fungi may therefore be related to the lack of ability of these fungi to produce such hyphal sheaths as efficiently as the white rot fungi. It is possible that in LNM and LNM with glucose and cell wall material there is a more evident target specificity. The brown rot fungi induce higher levels of enzymes than the white rot fungi, and this may be due to the ability of the latter to produce a hyphal sheath that is more effective in the presence of glucose, which is lost in LNM with only cell wall material. However there was a certain degree of target specificity in terms of higher levels of enzyme production against certain brown and white rot fungal cell wall materials. In both media containing cell walls laminarinase production was highest against cell walls of ***N.lepideus*** among the brown rot fungi and cell walls ***T.versicolor*** among the white rot fungi. It could be speculated from this that antagonism via lytic enzymes may be a more important antagonistic mechanism with respect to these two basidiomycetes than others. However, though chitinase showed the highest level of production with cell wall material of ***P.placenta*** among the brown rots in the absence of glucose the cell wall material of ***P.brevispora*** and ***T.versicolor*** induced the highest production among the white rot fungi. This lack of correlation in the degree of laminarinase and chitinase production against specific basidiomycete cell wall types suggests that the two enzymes are not equally important in the lytic activity against the same fungi.

This specificity of action of lytic enzymes is supported by the work of Sivan and Chet (1986) who showed that a ***T.harzianum*** isolate that failed to parasitise colonies of ***Fusarium oxysporum*** also failed to produce noticeable levels of laminarinase and chitinase when cell wall material of ***F.oxysporum*** was added to the media. However, the same antagonist produced higher levels of enzymes in the presence of the cell wall material of ***Rhizoctonia solani*** against which it was strongly mycoparasitic. So it is obvious that ***Trichoderma*** isolates exhibit target specificity against the pathogens.

The ***Trichoderma*** species that showed the highest level of laminarinase production in the presence of basidiomycete cell walls was ***T.pseudokoningii*** however ***T.viride*** isolate gave higher chitinase production. It is of interest to note that both these species were found to be the best inhibitors of both brown and white rot fungi in the interaction

studies (Chapter 2). In studies carried out by Dennis and Webster (1971 c) on hyphal interactions of many ***Trichoderma*** species with plant pathogenic fungi, the above mentioned two species were found to be the species which showed maximum coiling around pathogens. Results in medium containing glucose as well as cell wall material indicate however that ***T.aureoviride*** produces the highest levels of both laminarinase and chitinase. This particular species was included in the test as one of the controls as it did not inhibit either brown rot or white rot fungi in the LNM during interaction studies. And the high levels of enzyme seen probably represent the constitutive levels of enzyme involved in cell growth and autolysis, as the cell wall of ***Trichoderma*** are also made up of glucan and chitin. Levels of the enzymes produced by this species may be exaggerated compared to other isolates where they are carbohydrate repressed, while ***T.aureoviride*** shows no active repression.

Enzymic activity of glucosaminidase and galactosaminidase, the two enzymes that are involved in the further digestion of the cell wall materials were found to be produced at very low levels. Also the amount produced were extremely variable between the ***Trichoderma*** isolates and in the presence of different cell wall materials. However ***T.pseudokoningii*** spp. again seem to show higher levels of enzyme production as with laminarinase and chitinase. These two enzymes were however only produced in the medium containing cell walls but no glucose and were undetectable in the other two media. This indicates that perhaps these are more specialised enzymes produced only in the presence of the cell wall material or are subject to total catabolite repression by glucose. From the results it was also noted that basidiomycete cell wall material of ***G.trabeum*** showed the highest level of production of these enzymes. It can be speculated from this that these enzymes may only be involved in breakdown of some cell walls that have variable permutations of the glucan and chitin sugars in the wall especially, as the induction of these enzymes did not correlate well with laminarinase and chitinase.

In all the experiments carried out here and by other authors enzyme induction was achieved only in the presence of crude cell wall preparations of the target pathogens. However, if the whole process of mycoparasitism is to occur and the levels of both the

lytic enzymes likely to be produced ***in vivo*** is to be measured, mixed dual cultures of the antagonist and the pathogen need to be set up. Dual culture systems were set up by Freitag and Morrell (1992) with ***T.versicolor*** and ***T.harzianum***, but these authors only measured cellulase, cellobiose, laccase and peroxidase activity in these cultures. Such dual cultures could be set up however for measurement of laminarinase and chitinase. As chemotrophism and recognition of the pathogen are important stages in the process of mycoparasitism and are achieved due to detection of chemicals or certain glycoproteins or lectins as discussed earlier, it is more important to have the live pathogen in the growth medium with the antagonist than just the cell wall preparations. However if the cell wall extraction procedure has not undergone any protease treatment or any such protein removal step the cell wall material should still retain some proteinaceous material that may play a role in the recognition process by the antagonists.

Sivan and Chet (1986) in their study on inducibility of lytic enzymes by cell wall preparations of some plant pathogenic fungi, also observed that treatments of cell wall with 2N NaOH, protease or trypsin prior to their incubation with lytic enzymes of ***T.harzianum*** significantly increased the release of glucose and n-acetylglucosamine. These results suggest that proteins in cell walls of ***F.oxysporum*** may increase their resistance to degradation by extracellullar enzymes from ***T.harzianum***. Though removal of these proteins increases the susceptibility of the cell wall to the lytic enzymes, these same proteins may be responsible for the recognition stage between the antagonist and pathogen. Since the cell wall preparations used in this study may already be partially degraded due to grinding and sonication involved in the preparation stage, this may already have enhanced their susceptibility to lytic degradation. It is possible that during active mycoparasitism in nature small proteolytic enzymes may be produced to start breakdown and once the wall has been weakened after removal of some proteins, higher levels of lytic enzymes are produced. Elad ***et al.*** (1982) in their study of lytic enzyme inducibility by plant pathogenic cell wall material detected protease and lipase activity in the medium when antagonists attacked the mycelium of pathogens. It is also possible that the recognition process that has been said to involve lectins of the pathogen and sugar residues on the antagonist, could be active in this experiment to enable specific

recognition of wood decay fungi, by the ***Trichoderma*** species. The results indicate that some form of recognition is involved or there would not be any variation in the level of lytic enzymes produced with cell wall material of different wood decay fungi, as seen with the results presented here. This phenomenon may also account for the low levels of lytic enzyme production in the presence of laminarin and chitin alone in the medium as observed by Sivan and Chet (1986) and Elad ***et al.*** (1982), who found enzyme production was almost ten times higher in the presence of the cell wall material of the pathogenic fungi thereby indicating that the missing protein or specific sugar links on the laminarin and chitin must be responsible for the higher induction of the enzymes.

The results of this study show that ***Trichoderma*** species have the enzymic potential to exhibit mycoparasitism against wood decay fungi, judging from the levels of lytic enzymes produced and the inducibility by cell wall preparations of these basidiomycetes. However, this mechanism of antagonism is not necessarily the sole mechanism involved in the killing of the basidiomycetes. In the hyphal interaction studies of Murmanis ***et al.*** (1988 b) between ***T.harzianum*** and ***T.polysporium*** with wood decay fungi, they observed that in the scanning electron microscopy of the interactions of these fungi, even hyphae of basidiomycetes some distance away from the antagonists also showed denaturation of cytoplasmic material. These authors had already tested for soluble metabolite production by these organisms and had failed to show any such production, but speculated that such denaturation of fungal cell wall away from the interaction site may be due to production of inhibitory volatiles that can diffuse further, and produce effects away from the site of interaction. Therefore it is possible that volatiles and in other cases soluble metabolites may predispose the pathogen to further attack with lytic enzymes.

Chapter 3

Section 4

Siderophore Production

Chapter 3

Antagonistic Mechanism

Section 4 - Siderophore Production

3.4.1 Introduction

Iron is an indispensable nutrient for almost all living cells. It plays an essential role in respiration, nitrogen fixation, DNA and chlorophyll biosynthesis and other important enzymatic systems (Neilands, 1981 a). Although iron is one of the most common elements on Earth, its availability to living organisms is extremely low due to its insolubility. Under aerobic conditions iron is readily converted into its ferric form, which exhibits exceedingly high affinity for hydroxide ions to form insoluble polyhydroxide polymers. The solubility product constant of ferric hydroxide is 10^{-38} M, and at biological pH becomes 10^{-17} M which limits the concentration of free ferric ion to a value too low to sustain growth. Therefore, during the course of evolution, micro-organisms, plants and animals have developed efficient sequestering mechanisms for the ferric ion to ensure its utilisation (Neilands, 1981 a).

To cope with extreme iron deficiency, micro-organisms have developed high-affinity systems for ferric transport which consists of two components: a) the secretion of siderophores (Gr. = "iron bearers"), i.e., iron-regulated, low-molecular weight ferric-specific chelators, and b) the elaboration of membrane receptor molecules which bind the siderophores and transport them into the cell. Efficient non-siderophore systems may also exist in micro-organisms (Weinberg, 1989) and low-affinity systems operate under iron-sufficient conditions (Manulis ***et al.***, 1987 a; Neilands, 1981 a). The siderophore system is widely distributed in the microbial world, mainly among the aerobic and facultative anaerobic organisms. The molecular weight of the ferric complex of the usual siderophore lies in the range of 500 to 1500 daltons. Although considerable structural variation exists among the several dozen characterised siderophores (Neilands, 1981 a), the majority can be divided into two main classes, the hydroxamates and the catecholates (phenolates) (Hider, 1984). Bacteria are known to

produce both catecholate and hydroxamate type siderophores (Lankford, 1973) and until relatively recently fungi were thought to produce only hydroxamate siderophores (Neilands, 1984 a), however, production of phenolate type siderophores has been seen in fungi within the wood decay basidiomycetes (Jellison ***et al.***, 1990).

The hydroxamic acid siderophores and catechols contain, respectively one and three residues per mole of the chemical groupings -CO-N(OH)- or $C_6H_3(OH)_2CO$-. The prototypical example of the hydroxamate class is ferrichrome, a cyclic hexapeptide consisting of a tripeptide of glycine, a tripeptide of N^5-acetyl-N^5-hydroxyornithine, and one ferric ion. Virtually all fungi synthesise members of the ferrichrome class of siderophores, which are efficiently utilised by bacteria via specific receptor systems. The most common catechol-type siderophore is enterobactin, the product of the common enteric bacteria ***Escherichia coli*** and related bacteria. The hydroxamate/catechol classification has however been found to be too restrictive, since certain later discovered siderophore types could not be assigned to either of these two groups (Smith ***et al.***, 1985) eg., a novel complexon type siderophore (rhizoferrin) reported widely in the Mucorales (Winkelmann, 1992). Any typical siderophore has a binding constant for ferric ion of about 10^{30} M. Certain other trivalent metal ions, such as gallium and aluminium, are also firmly bound, as are some members of the actinide series. By comparison, divalent ions are only weakly complexed and this appears to be the mechanism whereby the iron is released into the interior of the cell (Winkelmann, 1990).

The genes and peptides involved in iron transport systems of fungi have not yet been elucidated, however on the basis of kinetic studies and structure-activity relationships combined with competition experiments, the mechanisms and specificity of the siderophore-mediated iron transport systems in fungi have been determined (Winkelmann and Huschka, 1987).

The different types of hydroxamate siderophores produced by fungi and their transport mechanism into the cell have been well documented (Winkelmann, 1990), however little is known of the phenolate or catecholate type of siderophores. A main structural

element of all fungal hydroxamate type siderophores is the amino acid orinithine which after N-hydroxylation and N-acylation gives an iron(III) complexing hydroxamic acid bidentate. Three main hydroxamate type fungal siderophore families are recognised, they are ferrichromes, cyclic triacetylfusarinines (triacetylfusigens) and coprogens including the dimerum acid and rhodotorulic acid subgroups. Ferrichromes represent a group of cyclic peptide siderophores with structural alterations in the peptide backbone and/or in the hydroxamic acid moieties. Some of the derivatives can be listed as follows - ferrichrome, ferricrocin, ferrichrome C, ferrichrysin, tetraglycyl-ferrichrome, ferrichrome A, ferrirubin and ferrirhodin. The cyclic triacetylfusarinines represent cyclic triesters of fusarinine residues possessing three ester bonds. The coprogens represent a group of linear trihydroxamic siderophores possessing both ester and peptide bonds. The dimerum acid is formed after cleavage of the ester bond in coprogen. The structure of rhodotorulic acid is similar to that of dimerum acid but the anhydromevalonic acid is replaced by acetic acid. A detailed review of the different hydroxamates isolated from different fungi and classified on the basis of the number of functional units present that bind the ferric iron can be found in Winkelmann, (1990).

The available data on fungal (hydroxamate) siderophore transport indicate that most fungal siderophores are transported as a whole across the plasma membrane, delivering iron to acceptors inside the cell and only in certain fungal genera do siderophores not penetrate the membrane barrier but rather deliver their iron to membrane bound acceptors. The latter mechanism has been termed iron taxi model. The taxi model was based on transport studies with ***Rhodotorula pilimanae*** using both tritium and radio labelled Fe-rhodotorulic acid (Carrano and Raymond, 1978). The pioneering work of Winkelmann, (1990) has identified many important aspects of the transport mechanism and specificity of siderophore uptake. It was found that irrespective of the actual mechanism involved, specific recognition of the appropriate siderophore by membrane components is essential to iron transport. And this specific recognition is dependent on the peptide side chains as well as the residues surrounding the metal centre in the siderophore. It has also been shown that membrane depolarisation significantly affects siderophore uptake which led to the conclusion that the membrane potential may be the actual driving force for siderophore translocation across the fungal plasma membrane

(Huschka *et al.*, 1983). It has also been noted by these authors that in many fungi the biosynthesis of a particular siderophore is linked to the recognition of that siderophore by receptors on cell membrane of those fungi. Therefore fungi have only been found to have receptor sites for fungal siderophores, however frequently bacteria possess multiple siderophore receptors, including receptors for fungal siderophores (Muller *et al.*, 1984).

The first researchers to note the production of phenolate siderophores by fungi were Jellison *et al.* (1990). These authors have shown that the brown-rot fungus ***Gloeophyllum trabeum*** can produce multiple chemically and serologically distinct metal chelators. HPLC purification resulted in the identification of several iron-binding peaks. Chemical analysis (Infrared IR and mass spectra MS and nuclear magnetic resonance NMR) of one of these compounds suggested the presence of a tri-substituted phenolate structure similar to the phenolate siderophores previously isolated by others from bacteria rather than the hydroxamate structure more commonly associated with fungal siderophores (Jellison *et al.*, 1991 b). The only information that is available on phenolate (catecholate) siderophores are from bacteria, which have phenol, catechol and/or 2-hydroxyphenyloxazoline as their ligand. Some of the catechols isolated to date can be listed as follows, agrobactin, parabactin, vibriobactin, pseudobactin, pyoverdine and pyochelin (Hider, 1984).

Siderophore production by ***Trichoderma*** species was only recently reported by Anke *et al.* (1991), but these authors however only reported production of hydroxamate type siderophores. They examined the production of siderophores by nine strains (six species) of the genus ***Trichoderma***. Under conditions of iron deficiency, the culture filtrate of all strains contained coprogen, coprogen B and ferricrocin. In addition, ***T.longibrachiatum*** and ***T.pseudokoningii*** produced siderophores of the fusigen type. They also extracted from the mycelia of some strains a new coprogen derivative which carried a palmitoyl instead of an acetyl group, and was named palmitoylcoprogen the first fungal siderophore which is solely found in the cells and not excreted in the culture broth.

Though siderophores are produced by virtually all micro-organisms the ability to sequester iron more efficiently is important for survival. This may be dependent on a number of factors: 1) different types of siderophores produced; 2) concentration of the siderophores produced; and 3) metal binding properties of the individual siderophores (Hider, 1984; Neilands, 1981 a; Crowley, 1991). All of the above factors will play a role in the competition for iron between organisms. As already mentioned, siderophores are virtually specific for Fe(III). This is achieved by the extraordinarily large thermodynamic formation constant for Fe(III) exhibited by these compounds, typically in the range of 10^{30} or higher. The siderophore ligands, whether hydroxamates or catechol, are exquisitely tailored for binding to the ferric ion. Since the chelating stability is critically dependent upon the ratio of charge/radius, the siderophore liganding atoms must be well-suited towards the ferric ion. As the latter is a "hard acid", it prefers to associate with a "hard base" such as oxygen and this accounts for the favouritism of $-O^-$ or -C=O groups in the coordination sphere of the iron. The stability constants with Fe(III) for certain hydroxamate and catechol type siderophores are listed in table 3.4.1. (Acetohydroxamic acid a synthetic siderophore is given for comparison and the results presented here on catecholates are only for bacteria).

Among the hydroxamic acids, it is apparent that the ferrichromes display a very modest chelating effect, a result which has been assigned to loss of energy through distortion of the binding (cyclohexapeptide) portion of the molecule (Hider ***et al.***, 1980). The very high value given for enterobactin is somewhat deceptive in that at neutral pH the apparent formation constant will be much closer to those shown for the hydroxamates. This can be attributed to competition for protons, the catechols being very weak acids (Raymond and Carrano, 1979). Even at pH 7.2, however, the catechols retain superior complexation capacity for iron (III) and it can be easily demonstrated that it can remove iron from ferrichrome (Corey and Bhattacharyya, 1977). An analog constructed from 1,3,5-tri-aminomethylbenzene was less powerful and appeared to equilibrate with ferrichrome iron. This suggests that there is something special about the 12-membered ring of enterobactin which makes it a perfect fit around the centrally coordinated metal ion (references in the above paragraph are as cited in Neilands, 1981 b).

Ligand	log Ks
HYDROXAMATES	
Deferriferrioxamine B	30.6
Deferriferrioxamine E	32.5
Deferriferriochrome	29.1
Deferriferrichrome A	29.6
Aerobactin	22.9
Rhodotorulic acid (dimeric/Fe)	31.2
Acetohydroxamic acid	28.3
CATECHOLATES	
Mycobactin	Exceeds deferrioxamine B
Enterobactin	52
Agrobactin/Parabactin	At neutral pH = enterobactin
Parabactin	Exceeds transferrin (iron transporter in blood)

Table 3.4.1 - Formation constants of siderophores with ferric iron at biological pH (Neilands, 1981 b).

Iron exchange between siderophores increases with time and is enhanced at low pH. Therefore iron exchange events render the interpretation of [^{55}Fe]-siderophore competition experiments difficult. However these difficulties can be overcome by using [^{14}C]-labelled siderophores. In addition, iron exchange events are minimised by using a non-siderophore producing strain. This was achieved (Winkelmann, 1973) using a mutant of ***Neurospora crassa*** (arg-5 ota aga), which cannot biosynthesis ornithine, a necessary constituent of most fungal siderophores. This mutant is therefore unable to biosynthesise siderophores, if ornithine is omitted in the cultivation medium. It was shown that there is competition between [^{55}Fe]-ferricrocin and [^{14}C]-coprogen during uptake by ***N.crassa*** (arg-5 ota aga). [^{14}C]-coprogen uptake rates are decreased in the presence of increasing amounts of [^{55}Fe]-ferricrocin and vice versa. This experiment is proof that siderophore competition occurs during uptake by fungal cells, however it does not allow determination of the number of transport systems involved during siderophore uptake. Competition for one uptake system does not exclude the existence of a second uptake system (Winkelmann, 1973).

Due to such differences in affinity and production of siderophores by different fungi, iron competition can play an important role in a number of combative situations. Competition for iron is a way of life for soil micro-organisms and exerts a profound effect on the interactions among saprophytic microbes, pathogens and plants. The involvement of siderophores produced by ***Pseudomonas*** species in suppressing soil-borne diseases is documented in numerous reports and reviews (Leong, 1986; Leong and Expert, 1989; Neilands and Leong, 1986; Schroth and Hancock, 1982). The suppression phenomenon is not only limited to fungal and bacterial phytopathogens, but also affects deleterious rhizobacteria (Suslow and Schroth, 1982). The possible role of siderophores as virulence determinants of plant pathogens has been the subject of investigations for sometime (Leong and Expert, 1989; Neilands and Leong, 1986). In some diseases the iron status of the host plant has been shown to exert a significant effect on the course of infection (Barash ***et al.***, 1988; Guerra and Anderson, 1985). In addition, siderophores may affect spore germination of fungal pathogens (Swinburne, 1986).

It is evident that siderophores play an important role in the biocontrol of many plant pathogenic fungi and bacteria (Neilands, 1984 a). But to date little has been published regarding iron competition between ***Trichoderma*** and wood decay basidiomycetes in wood as a possible antagonistic mechanism. It has been known for sometime that hydroxamate type siderophores are produced by basidiomycetes. But phenolate type siderophore production by these fungi was only recently reported by Jellison ***et al.*** (1990) and was thought to be unique to wood decay basidiomycetes. The production of hydroxamate type siderophores by ***Trichoderma*** species has also only recently been reported by Anke ***et al.*** (1991).

If phenolate type siderophores are also produced by ***Trichoderma*** and the high affinity of phenolates for many metals may exhibit competition for the iron with the siderophores of basidiomycetes, as they are likely to have an equal potential for sequestering these metal ions. However the individual siderophore-Fe affinity as determined by ligand type and the concentration of siderophores produced will both

hydroxamate siderophore produced by the wood decay fungi and ***Trichoderma***, may determine which produce the more efficient iron sequestering agents. This will have important implications in terms of iron competition between the organisms as it is partly dependent on the chelating ability of the siderophores. Such characterisation studies require the application of many chromatographic and spectroscopic techniques. The two chosen techniques in this study were that of Thin Layer Chromatography (TLC) to identify the number of individual siderophores; and nuclear magnetic resonance (NMR) to provide information on the chemical nature of the ligand groups. Complete characterisation of the chemical structure of the siderophore compounds is however a difficult task as they may contain up to 41 carbon and 64 hydrogen atoms like eg., ferrirubin. Carbon and proton NMR of these compounds do not allow easy interpretations of the functional groups due to their complicated bonding patterns that will affect the NMR spectra. Nevertheless the important functional groups that dominate the characteristics of the siderophores like the presence of an aromatic group (phenolate) can be elucidated. Mass spectra and Infrared spectra studies are however needed for complete elucidation of the siderophore structure.

This chapter examines the potential role of ***Trichoderma*** siderophores in the biocontrol of wood decay fungi.

3.4.2 Materials and Methods

3.4.2.1 Interaction studies on LNM (with reduced iron)

Interaction studies were carried out on the low nutrient media with the same nutrient consistency as described in chapter 2 and LNM with reduced concentration of iron. In the original LNM, $FeSO_4$ is present as the sole source of iron at a concentration of approximately 65.7 µM of iron i.e., 0.0183 g/l. However in the low iron media the $FeSO_4$ concentration was reduced to 0.65 µM of iron (0.000139 g/l) as siderophore production is reported to be induced (Neilands, 1984 a) only when the level of iron concentration is low (0.1 to 10 µM). Interaction studies were carried out with the ten selected ***Trichoderma*** isolates against the two basidiomycetes ***N.lepideus*** FPRL 7F and ***T.versicolor*** MAD 697. The method of study and analysis of the results for the interaction studies are as described in chapter 2. All the glassware used in the experiment were soaked overnight in 0.05 M EDTA (Ethylenediaminetetraacetic acid - Sigma E4884) and thoroughly washed at least three times with deionised distilled water. All the media were also prepared in deionised water. Such precautions were essential to keep the contamination by minerals, especially iron to a very low level as these may interfere with the test. In all the experiments that follow the above precautions were observed.

3.4.2.2 Chemical Assay for Detection of Siderophores - CAS (Chrome azurol S) agar medium

The production of these low molecular weight, proteinaceous molecules was determined by a detection method based on their high affinity for Fe III. The ternary complex chrome azurol S (CAS)/iron III/hexadecyltrimethylammonium bromide serves as an indicator. When a strong chelator such as a siderophore removes the iron from the dye, its colour changes from blue to orange. When this CAS complex is incorporated into agar plates, orange halos around the colonies are indicative of siderophore excretion.

Plates of CAS agar (Schwyn and Neilands, 1987) were prepared as follows. To prepare 1 litre of blue agar, 60.5 mg CAS (Fluka Biochemicals, Packed in Switzerland) was dissolved in 50 ml of water and mixed with 10 ml Fe (III) solution (1 mM $FeCl_3 . 6H_2O$, 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg HDTMA

(Hexadecyltrimethylammonium bromide - Fluka) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved (solution A). To 750 ml of H_2O, was added MM9 salts (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl dissolved in 100 ml of water, the pH adjusted to 7.4, to this was added separately 0.493 g of $MgSO_4$ in 0.2 ml of water, 2 g of glucose in 1 ml water and 0.011 g of $CaCl_2$ in 0.01 ml of water); also added were 15 g agar, 30.24 g Pipes (1,4-piperazinediethanesulfonic acid - Fluka), and 12 g of a 50% (w/w) NaOH solution to raise the pH to the pKa of Pipes (6.8) and autoclaved for 15 min at 121 °C. After cooling to 50 °C, 30 ml casamino acids (10%) (Difco), acting as the carbon source, was added (solution B). The dye solution (solution A) was finally added to solution B along the glasswall, with enough agitation to achieve mixing without generation of foam. Each plate received 30 ml of blue agar. Plates of CAS were inoculated separately with cores of each of the ten selected ***Trichoderma*** and the two basidiomycetes ***N. lepideus*** and ***T.versicolor*** (0.6 cm in diameter) and incubated at either 22 or 25 °C. After an incubation period of one week the CAS plates were examined for orange halos, and the diameter of the halos and their colours were noted.

CAS agar with MEB or LNM Nutrients

The CAS agar was modified by replacing the nutrients of the medium i.e., the MM9 salts and casamino acids with malt extract broth (MEB) or LNM (with the exclusion of iron). Results were recorded as above.

3.4.2.3 Isolation and Purification of Siderophores

All the solvents and chemicals used in the extraction and purification procedures are of analar grade obtained from either Sigma or Aldrich, Dorset, England.

The following three isolates of ***Trichoderma*** were chosen for extraction of the hydroxamate and phenolate siderophores, ***T.harzianum*** 25, ***T.pseudokoningii*** 64, and ***T.viride*** 60. They were chosen on the basis of their antagonistic responses against the basidiomycetes tested and because they represented different species. The three ***Trichoderma*** isolates were grown in 6 liters of the LNM (autoclaved at 121 °C) without any added iron in EDTA washed glassware. The inoculum consisted of three plugs of

Trichoderma mycelium (0.6 cm in diameter) taken from a 3 % MEA plate. This transfer of the organisms from a high iron media (MEA) to a low iron medium (LNM-Fe) was undertaken to stress the organism and induce production of siderophores. Cultures were incubated at 22 or 25 °C for about 3 weeks until the fluorescent yellow colouration (thought to be indicative of siderophore production) was evident in the spent culture medium. The spent culture medium was filtered to remove the mycelium by passing through a buchner funnel with Whatman No. 1 filter paper. This was subsequently passed through a 0.45 µm sterile membrane filter (Whatman) and stored at 4 °C. The supernatant fluid was then concentrated to 1/5 th the original volume by rotary evaporation at 45 °C. The concentrated culture supernatant from each ***Trichoderma*** isolate was then divided into equal volumes for extraction of the two types of siderophores.

Isolation of Hydroxamate Siderophores

Ferrous sulphate (hydrous) was added to the concentrated supernatant at a concentration of 1 mg / ml (the Fe added binds to the siderophores present giving it a bright yellow/orangey colour). The solution was then acidified with conc HCl to pH 3 and $(NH_4)_2SO_4$ was added while stirring in a beaker with a magnetic flea until the solution was saturated. This solution was then extracted several times in a separating funnel (3 to 4 times) with small aliquots of benzyl alcohol (1/10 to 1/20 the volume of supernatant) (Garibaldi and Neilands, 1954). The disappearance of the characteristic colour of the siderophores from the aqueous layer indicated that the extraction of the siderophores from the culture supernatant into the benzyl alcohol was complete. The alcohol extracts were combined and the hydroxamates partitioned into water by addition of 3 volumes of diethyl ether and 1/10 volume of water. The aqueous extracts were combined and then washed with more diethyl ether to remove any traces of benzyl alcohol present (Fekete ***et al.***, 1989). The washed aqueous extracts were then rotary evaporated at 45 °C to dryness and the residual material resuspended in a small volume of methanol (3 to 5 ml).

The concentrated siderophores in methanol were then purified by passing through a Normal Phase Sep-Pak (Light) NH_2 Cartridge (Millipore, Waters Division, Chicago,

U.S.A). These cartridges are specifically used for purification of metabolites where excessive dilution is a concern and these cartridges contain two-third less sorbent than the standard Sep-pak cartridge and give higher sample recovery with less solvent consumption. The general elution protocol for Normal Phase Chromatography involved conditioning of the cartridges with 2 ml of a polar solvent, (methanol) followed by 5 x 2 ml water to hydrate. Immediately after, the samples were passed through and the siderophores in methanol retained in the cartridge as indicated by their colour while unwanted contaminating components passed through. The cartridge was then washed through with 5 to 8 ml of water to remove the excess salts and other impurities. This was then followed by elution of the siderophores by passing through 5 ml of methanol and the release of the siderophores was indicated by the elution of the colour passing down the column into the collection vial. The purified sample was then stored at 4 °C for further studies.

Isolation of Phenolate Siderophores

The concentrated culture supernatant for each isolate was acidified with conc HCl to pH 3.0. This was followed by solvent extraction with an equal volume of ethyl acetate. This was repeated twice more before the combined ethyl acetate extracts were evaporated to dryness in a rotary evaporator with a 40 °C water bath and the residue resuspended in a small amount (2 to 3 ml) of ethyl acetate (Jellison ***et al.***, 1991 a). The procedures carried out for purification of the phenolates with the Sep-pak cartridges (NH_2) were similar to that for the hydroxamates. The cartridges were conditioned in the same way as before with methanol, and the samples in ethyl acetate passed through while the impurities were washed out with more methanol (2 to 3 ml). Then the siderophores were eluted out with small volumes of (2 to 3 ml) a non-polar solvent, chloroform.

Thin Layer Chromatography (TLC) of the Siderophores

Fifty microliters (µl) volumes of the purified siderophores, (hydroxamates and phenolates) were dotted onto silica gel plates and a cold air blower used to dry the samples. The samples were then separated by TLC on a 250 µm silica gel plate (Analtech, Chicago, U.S.A). The solvent system used for TLC of hydroxamates was a solvent mixture of chloroform:ethanol:water (80:19:1) (Bentley ***et al.***, 1986) and for

phenolates a mixture of chloroform:ethyl acetate:formic acid:toulene (40:40:10:10) (Jellison *et al.*, 1991 a). The purified samples (hydroxamates in methanol and phenolates in ethyl acetate) were then allowed to run in the appropriate solvent mixtures in a closed glass tank. The silica plates were then allowed to dry. Detection of the hydroxamates was simply done by drying in an oven at 37 °C for half an hour and the siderophores detected by the yellowish orange dots. The phenolic compounds were visualised under UV light (short wavelength) and detected as fluorescent spots.

3.4.2.4 Nuclear magnetic resonance (NMR) of the purified siderophores (hydroxamates and phenolates)

NMR experiments were performed with a Bruker WH250 spectrometer operating at 63 MHz for ^{13}C and at 250 MHz for proton ('H) detection. The purified hydroxamate and phenolate siderophores were dissolved in $CDCl_3$ (deuterated chloroform), CD_3OD (deuterated methanol) or D_2O (deuterated water) as listed in table 3.4.2.4. The liquid state NMR was done with 0.5 ml volumes of the respective solvents with non TLC separated purified siderophore samples. And all spectra were run at 20-22 °C.

Solvents	Trichoderma Iso.	^{13}C	Dept	'H
$CDCl_3$	B11 P	x		x
$CDCl_3$	B15 P	x	x	x
$CDCl_3$	R10 P	NT		x
CD_3OD	B11 H	x		x
CD_3OD	B15 H	NT		x
D_2O	R10 H	x		x

Table 3.4.2.4 - Proton ('H) and Carbon (^{13}C, Dept) NMR carried out (x) on the Hydroxamate (H) and Phenolate (P) extracts of the three ***Trichoderma*** isolates B11 (***T.harzianum***), B15 (***T.pseudokoningii***) and R10 (***T.viride***). NT - Not tested.

The proton and carbon NMR could not be carried out on all the extracts as the amounts of sample were not sufficient, and table 3.4.2.4 lists the types of NMR analysis undertaken for each of the samples. TMS (tetramethylsilane) was used as the reference with all the solvents except water, DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate) was used as reference with D_2O as the former is insoluble in water.

3.4.3 Results

3.4.3.1 Interaction studies on LNM and LNM (with reduced iron)

The results of the interaction studies carried out with the ten selected ***Trichoderma*** isolates against the brown rot fungus ***Neolentinus lepideus*** and the white rot ***Trametes versicolor*** are shown on table 3.4.3.1. It is clear that outcome of interactions between antagonists and decay fungi were not always reproduced in the two media types (LNM and LNM with reduced iron). B1 (***T.aureoviride***) shows the same results in both media where neither basidiomycete are killed. B13, B15, (***T.pseudokoningii***) and R10 (***T.viride***) show elimination of both wood decay fungi in both LNM+reduced iron and LNM. However in many interactions the apparent rate of death and lysis of the brown rot ***Neolentinus lepideus***, as evident from the extent of browning, increased in the low iron medium (Figures 3.4.3.1 a and b). The rest of the isolates tested seemed to show some varied results. In interactions with the white rot fungus, however no browning was evident at or near the interaction zone with any of the ***Trichoderma*** isolates. Figures 3.4.3.1 (c) and (d) show the interaction of isolates B13 and B15 (***T.pseudokoningii***) with the two wood decay fungi. As seen with isolate B11 (***T.harzianum***) against the white rot fungus there is no correlation between the production of browning and death in the two basidiomycetes (Figures 3.4.3.1 b).

Trichoderma Isolates	Low Nutr. Med.+Fe		Low Nutr. Med.+Fe*	
	Nl	Tv	Nl	Tv
B1	S	S	S	S
B5	K	K	S	K
R2	S	K	S	K
R7	K	K	S	S
R10	K	K	K	K
B11	K	K	K	S
B13	K	K	K	K
B14	K	K	K	S
B15	K	K	K	K
B16	K	K	S	S

Table 3.4.3.1 - Outcome of interaction studies between ***Trichoderma*** isolates and the two basidiomycetes. K=complete death of the decay fungus; S=Survival of the decay fungus; * reduced concentration of iron. Basidiomycetes, Nl = ***Neolentinus lepideus*** and Tv = ***Trametes versicolor***. ***Trichoderma*** isolates; B1 = ***T.aureoviride***; B5, R2, R7 and R10 = ***T.viride***; B11 = ***T.harzianum***; B13, B14 and B15 = ***T.pseudokoningii*** and B16 = unidentified ***Trichoderma*** species.

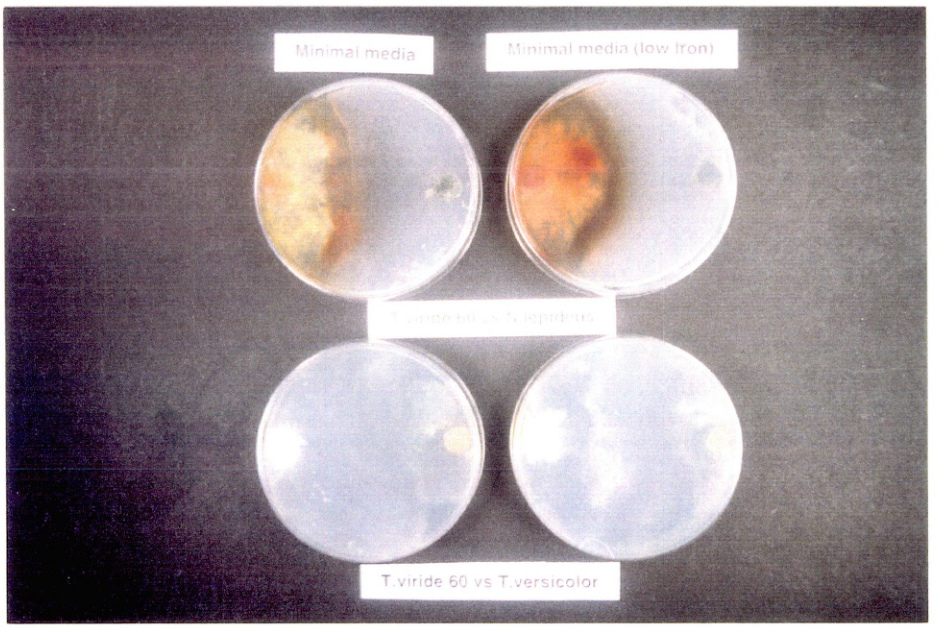


Figure 3.4.3.1 (a) - Interaction plates between ***T.viride*** 60 (R10) and, ***Neolentinus lepideus*** and ***Trametes versicolor*** on LNM (left) and LNM+reduced iron (right). The basidiomycetes were inoculated on the left sides of the plates and the ***Trichoderma*** isolates on the right side.

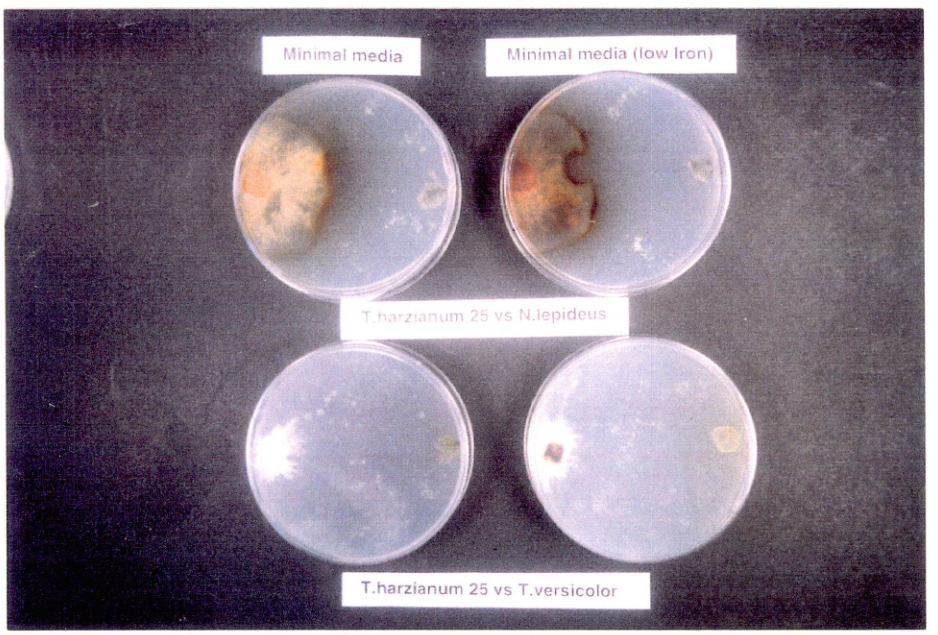


Figure 3.4.3.1 (b) - Interaction plates between ***T.harzianum*** 25 (B11) and ***Neolentinus lepideus*** and ***Trametes versicolor*** on LNM (left) and LNM+reduced iron (right). The basidiomycetes were inoculated on the left sides of the plates and the ***Trichoderma*** isolates on the right side.

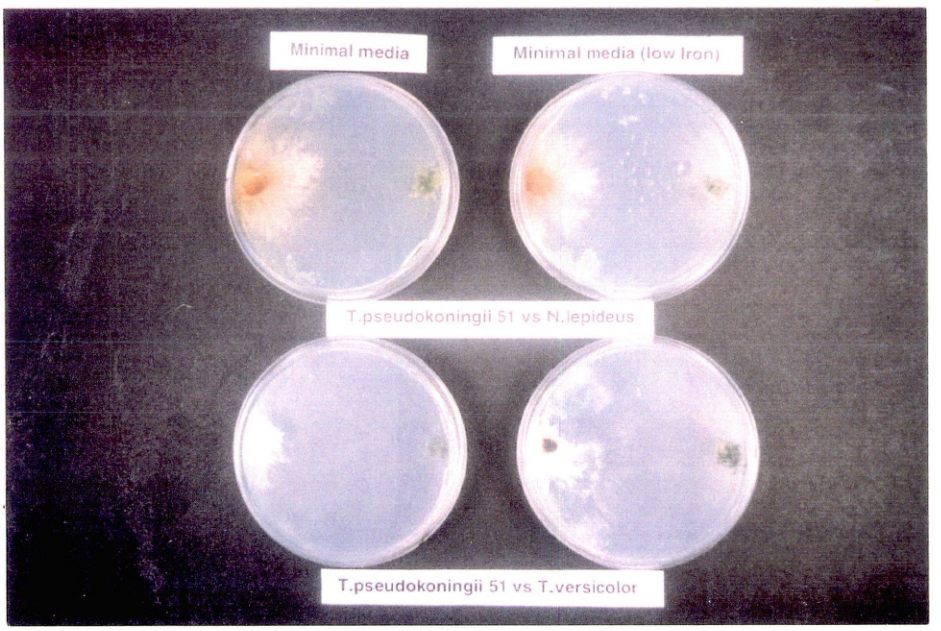


Figure 3.4.3.1 (c) - Interaction plates between ***T.pseudokoningii*** 51 (B13) and, ***Neolentinus lepideus*** and ***Trametes versicolor*** on LNM (left) and LNM+reduced iron (right). The basidiomycetes were inoculated on the left sides of the plates and the ***Trichoderma*** isolates on the right side.

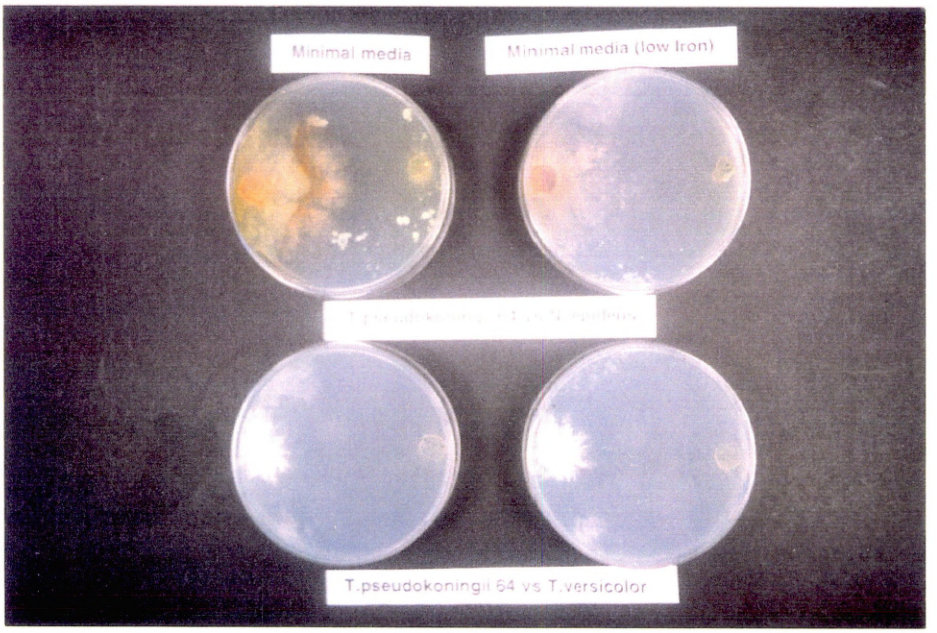


Figure 3.4.3.1 (d) - Interaction plates between ***T.pseudokoningii*** 64 (B15) and, ***Neolentinus lepideus*** and ***Trametes versicolor*** on LNM (left) and LNM+low iron (right). The basidiomycetes were inoculated on the left sides of the plates and the ***Trichoderma*** isolates on the right side.

3.4.3.2 Chemical assay for detection of siderophores - CAS (Chrome azurol S) agar medium and CAS agar with MEB or LNM nutrients

Table 3.4.3.2 shows the extent of growth, halo size and colour as for all ten selected ***Trichoderma*** isolates and the two basidiomycetes grown on CAS, CAS+MEB (malt extract broth) and CAS+LNM (low nutrients medium). It is clear from the results in CAS+MEB that none of the fungi produce any siderophores in this medium. This is likely to be due to the fact that malt extract broth contains very high concentrations of iron and siderophores are only induced when iron level is low. Although fungal growth is smaller in both CAS and CAS+LNM, siderophore production by some isolates is much greater in the CAS compared to that of the CAS+LNM. Only ***T.aureoviride*** isolate B1, produced no siderophores in any of the three media types (figure 3.4.3.2 (a)). Within the ***T.viride*** species (B5, R2, R7 and R10), all produced siderophores in the CAS medium, but in the CAS+LNM medium the halo size was reduced for isolates R2, R7 and R10 (figure 3.4.3.2 (b)) and not produced with B5. The colour of the halos however remain the same in the two media. ***T.harzianum*** isolate B11, shows the highest amount of growth and the largest halo size of all isolates in CAS and CAS+LNM media (figure 3.4.3.2 (c)). The colour of the halo however is very distinctive from the rest and is fluorescent yellow appearance. Isolates B13 (figure 3.4.3.2 (d)), B14 and B15 (figure 3.4.3.2 (e)) ***T.pseudokoningii***, show measurable growth and halo size in the CAS medium, but produce no siderophores in the CAS+LNM. These species show an orangey/yellow halo colour like that of ***T.viride*** R7. The unidentified ***Trichoderma*** B16 shows similar responses to that of the ***T.pseudokoningii*** isolates. The two basidiomycetes (Tv (figure 3.4.3.2 (f) and Nl) also show siderophore production but the halos were as not well defined as in the ***Trichoderma*** isolates. They do however produce a much deeper reddish/orange colouration than the ***Trichoderma*** isolates. This was observed for both the fungi on CAS and CAS+LNM.

Trichoderma Isolates	CAS medium			CAS+MEB medium			CAS+LNM medium		
	Growth	Halo	Colour	Growth	Halo	Colour	Growth	Halo	Colour
B1	++	-	-	++++	-	-	++	-	-
B5	++	++	orange	++	-	-	++	-	-
R2	+/-	+++	orange	+/-	-	-	+/-	+	orange
R7	+/-	+	or/ye	+/-	-	-	+/-	+/-	or/ye
R10	+/-	++++	orange	+/-	-	-	+	+++	orange
B11	+++	+++++	flu/ye	+++	-	-	+++	++++	flu/ye
B13	+	++	or/ye	+	-	-	+	-	-
B14	+++	+	or/ye	+++	-	-	+++	-	-
B15	++	++	or/ye	++	-	-	++	-	-
B16	++	++	or/ye	+	-	-	+++	-	-
Tv	+	++	red/or	+	-	-	+	++	red/or
Nl	+	++	red/or	+	-	-	+	++	red/or

Table 3.4.3.2 - The extent of growth, halo size and colour of the selected ten ***Trichoderma*** isolates and two basidiomycetes in CAS, CAS+MEA and CAS+LNM agar. Key :- Increasing (+, ++, +++...) correspond to increase in growth of fungi or halo size; (-) indicates no growth or halo; or - orange, ye - yellow, flu - fluorescent, represent halo colouration. Basidiomycetes, Nl = ***Neolentinus lepideus*** and Tv = ***Trametes versicolor***. ***Trichoderma*** isolates; B1 = ***T.aureoviride***; B5, R2, R7 and R10 = ***T.viride***; B11 = ***T.harzianum***; B13, B14 and B15 = ***T.pseudokoningii*** and B16 = unidentified ***Trichoderma*** species.

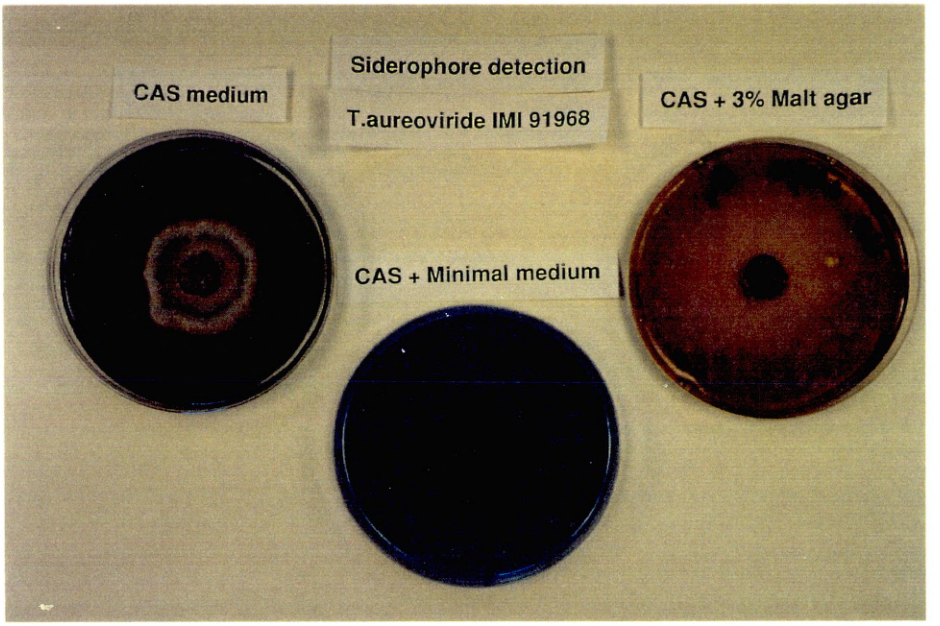


Figure 3.4.3.2 (a) - Siderophore production by *T.aureoviride* IMI91968 (B1) on CAS, CAS+MEB and CAS+LNM. Siderophore production is absent in all media in this case.

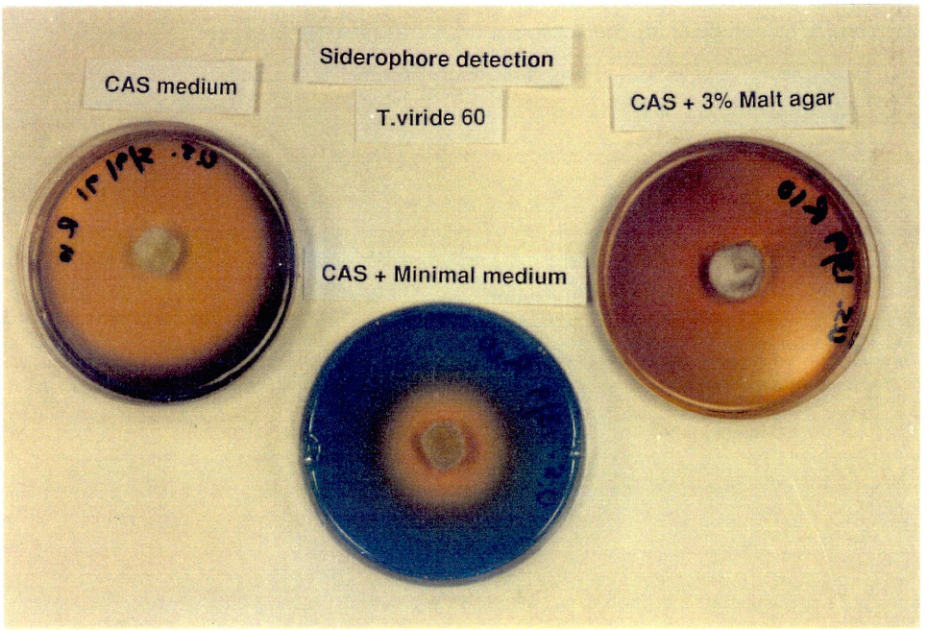


Figure 3.4.3.2 (b) - Siderophore production by ***T.viride*** 60 (R10) on CAS, CAS+MEB and CAS+LNM. Siderophore production is indicated by the halos around the fungal cores. Note the orange colouration of the halos only around the core in CAS and CAS+LNM.

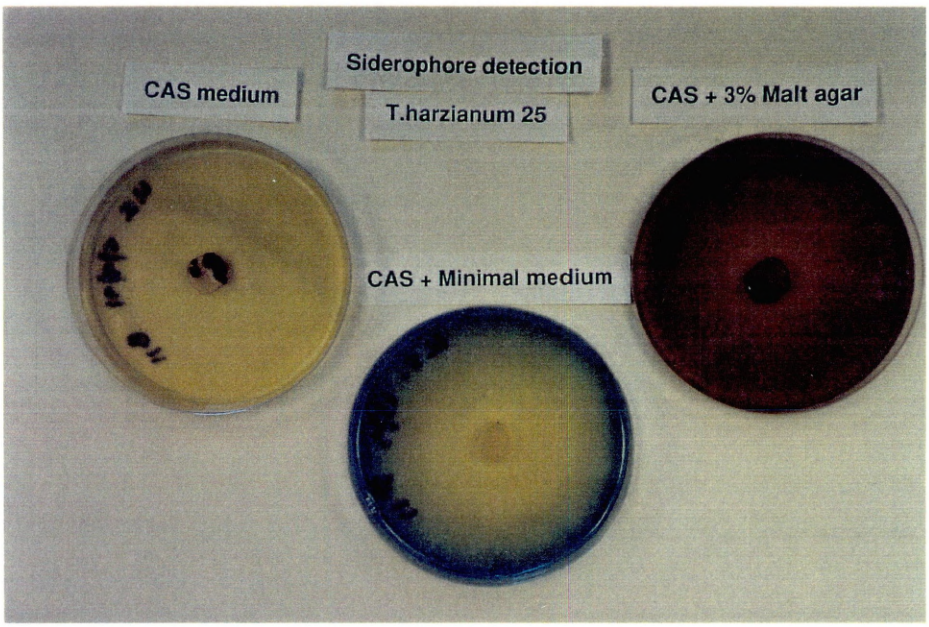


Figure 3.4.3.2 (c) - Siderophore production by ***T.harzianum*** 25 (B11) on CAS, CAS+MEB and CAS+LNM. Siderophore production is indicated by the fluorescent yellow colouration underneath and around the mycelium in CAS and CAS+LNM but absent in CAS+MEB.

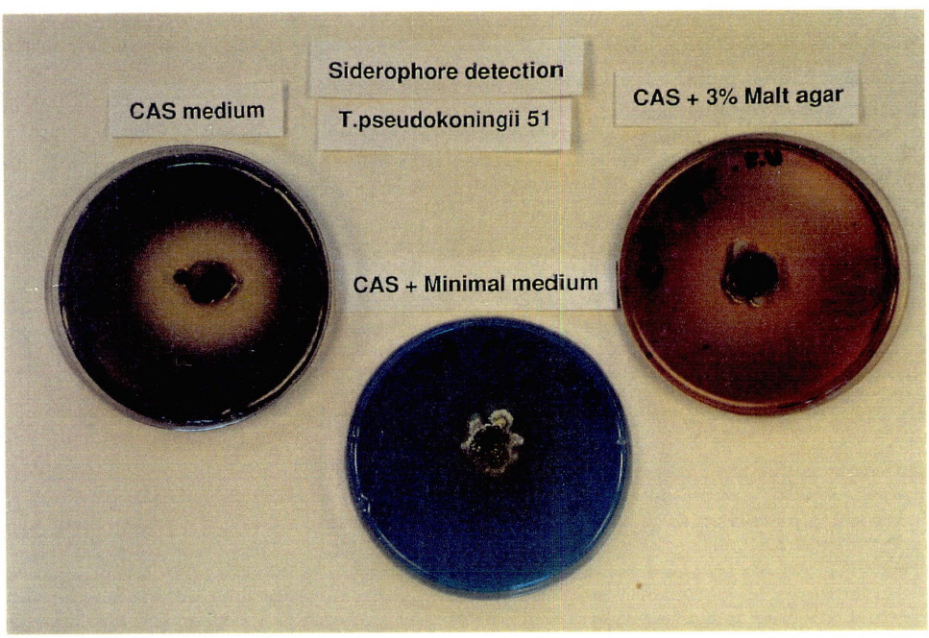


Figure 3.4.3.2 (d) - Siderophore production by ***T.pseudokoningii*** 51 (B13) on CAS, CAS+MEB and CAS+LNM. Siderophore production is indicated by the halos around the fungal cores. Note the orangey/yellow colouration of the halo around the core in CAS but absent in both CAS+LNM and CAS+MEB.

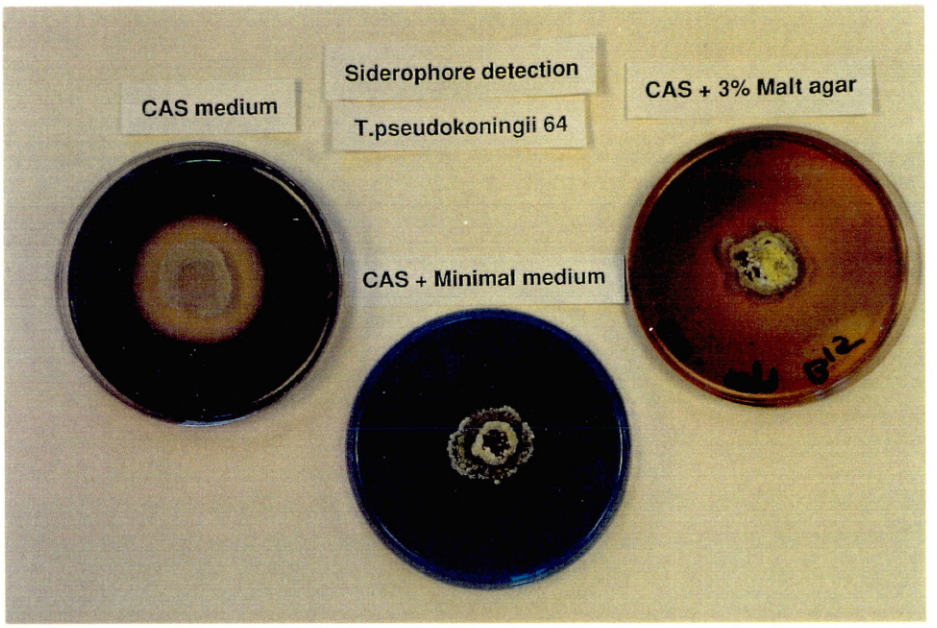


Figure 3.4.3.2 (e) - Siderophore production by ***T.pseudokoningii*** 64 (B15) on CAS, CAS+MEB and CAS+LNM. Siderophore production is indicated by the halos around the fungal cores. Note the orangey/yellow colouration of the halo around the core in CAS which is more defined than that of B13. The halo is absent in both CAS+LNM and CAS+MEB.

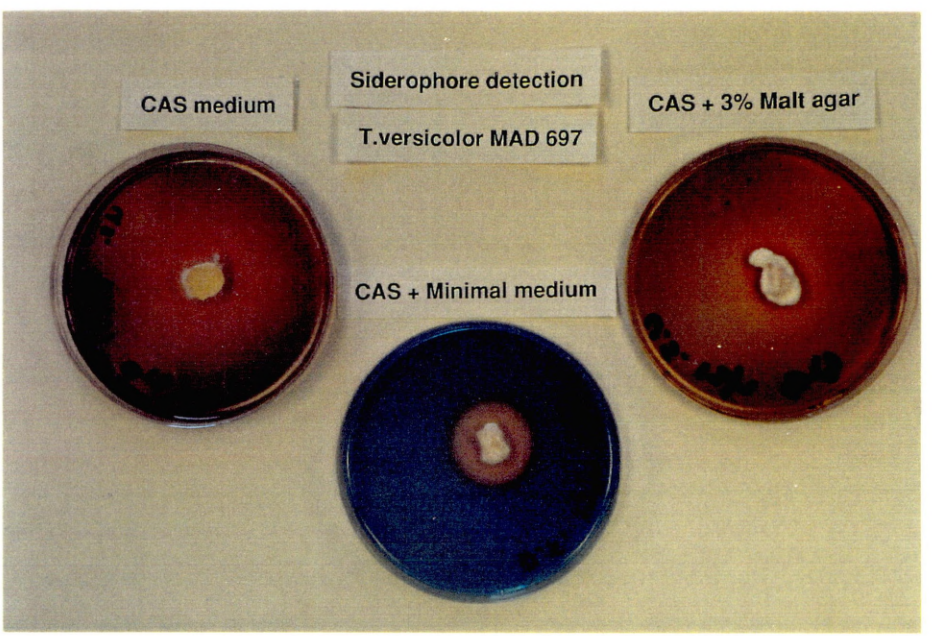


Figure 3.4.3.2 (f) - Siderophore production by ***Trametes versicolor*** on CAS, CAS+MEB and CAS+LNM. Siderophore production is indicated by the halos around the fungal cores. Note the reddish/orange colouration of the diffused halo around the core in CAS and the more defined halo in CAS+LNM. No halo is present in CAS+MEB.

3.4.3.3 Isolation and purification of siderophores - TLC of purified siderophores

Hydroxamate and phenolate type siderophores were extracted from the three Trichoderma isolates R10, B11 and B15 and were then purified and stored at 4 °C in their respective extraction solvents (Hydroxamates in methanol and Phenolates in chloroform). The results of thin layer chromatography (TLC) of the purified siderophore are presented in table 3.4.3.3. It is evident from the Rf values obtained that the hydroxamate siderophore compounds are immobile which suggests that the siderophores detected may be either of Ferrichrome A or Coprogen B type (Konetschny-Rapp ***et al.***, 1988; Budde and Leong, 1989). These TLC spots could however have both of the hydroxamate type siderophores in them as the Rf values on silica gel are the same for the two siderophores. Anke ***et al.*** (1991) also reported that different species and strains of ***Trichoderma*** also produce Ferrichrome A and Coprogen B type siderophores.

The Rf values of the R10 and B11 phenolates indicate that these isolates only produce a single but different phenolate type siderophore. However B15 (***T.pseudokoningii***) produces two phenolate types one of which would appear to be similar to that produced by B11 (***T.harzianum***), as indicated by the Rf values. Comparison of the Rf values of a phenolate type siderophore of the brown rot fungus ***Goleophyllum trabeum*** (Jellison ***et al.***, 1991 a) with that obtained here for ***Trichoderma***, suggests that B11 and B15 may be producing a phenolate type siderophore similar to that of the brown rot fungus. The Rf values of the siderophore extracts from both wood and liquid media of ***Gloeophyllum trabeum*** showed a doublet band at 0.88 (Jellison ***et al.***, 1991 a). A similar doublet band was recorded in this study with B15 and a single band with B11 at Rf = 0.86 which is very close to that reported by Jellison ***et al.*** (1991 a) for ***G.trabeum***. B15 (***T.pseudokoningii***) and R10 (***T.viride***) also showed another set of bands at 0.39 and 0.017 respectively.

Trichoderma Isolates	Hydroxamates	Phenolates
R10	0.0	0.017 cm
B11	0.0	0.86 cm
B15	0.0	0.39, 0.86* cm

Table 3.4.3.3 - Rf values of TLC bands of hydroxamates and phenolates of *Trichoderma* isolates R10 (***T.viride***), B11 (***T.harzianum***) and B15 (***T.pseudokoningii***). * indicates an unusual double band at the same reference point.

3.4.3.4 Nuclear Magnetic Resonance - Carbon and Proton Spectra

Table 3.4.2.4 lists the proton and carbon NMR analysis for with hydroxamate and phenolate extracts of the three ***Trichoderma*** isolates B11, B15 and R10. Analysis of the spectra for the hydroxamate (H) siderophores for the three antagonists, (Appendix II) indicates that it is possible that B11 and B15 may produce a ferrichrome A or coprogen B type siderophore, however the spectra of R10 shows the absence of any similar signals. The low amounts of sample available produced poorly resolved spectra and any assignments must be considered tentative. The proton spectrum of B11 (H) was well resolved and the spectrum indicates the presence of large amounts of aromatic CH, as indicated by peaks between 7.2 and 7.6 ppm (parts per million). There is a peak at 1.3 that is indicative of C-CH protons while the peaks at 3.2 ppm indicate the possibility of nitrogen associated groups. The carbon spectrum of B11 (H) supports the proton spectrum in concluding that there are aromatic groups in the compound (at 130 and 142 ppm). There is also a possibility of a C=O (ketone) group at 160 ppm, and between 60 and 100 ppm there is the possibility of saturated carbons attached to alkyl groups (CH). The proton spectrum of B15 (H) however shows more noise or interference. There appears to be some aromatic CH as indicated by peaks at 7.5 ppm and also an alkyl singlet at 1.3 ppm and a large singlet at 3.2 indicating a CH-O bonding. No carbon spectrum could be obtained for B15 (H) due to small quantity of compound.

With the phenolate (P) siderophore spectra it could be concluded that B11 (P) and B15 (P) are very similar, indicating that they might be producing the same type of

siderophore as suggested by the TLC results where both B11 and B15 showed bands at Rf = 0.86. The spectra obtained for R10 also indicate presence of a different phenolate type siderophore.

The proton spectrum of B15 (P) indicated the presence of some alkyl groups between 1 to 2 ppm and possible presence of a monosubstituted phenol group between 6.5 and 7.5 ppm. This is also supported by the carbon spectrum where three peaks at 123, 124 and 143 ppm show the presence of a phenol group. The presence of the CH_3 and CH_2 groups can also be seen around 20 and 40 ppm. DEPT (Distortionless Enhancement by Polarisation Transfer) carbon spectra was done to confirm the presence of these CH_2 and CH_3 groups, which can be seen as -ve and +ve peaks respectively on the spectrum. The proton and carbon spectra of B11 (P) are identical to that of B15 (P). Only a proton spectra could be obtained with R10 (P) due to insufficient amount of sample, however it indicated that there are possibilities of a phenolate group in the siderophore molecule.

Obviously other characterisation techniques (MS and IR) would need to be employed to fully determine the structure of these siderophore compounds. Also the spectrum were more difficult to interpret since the NMR spectra were that of a combination of each of the hydroxamate and phenolate type siderophores, as produced by any one ***Trichoderma*** isolate, as separation of individual bands from TLC would mean even less amounts of sample. But on the basis of the limited study carried out here with TLC and NMR, it is possible to say that the three ***Trichoderma*** isolates tested produce both hydroxamate and phenolate type siderophores. The hydroxamate types could be grouped under the Ferrichrome A or Coprogen B type siderophores. And the phenolates produced by at least two of the isolates (B11 and B15) show possibilities of production of the same type of siderophores as that of the brown rot fungus ***Gloeophyllum trabeum.*** In addition they also seem to produce other types of phenolates that do not resemble the ones produced by the brown rot fungus which may or may not be better chelating agents than the ones produced by wood decay fungi.

3.4.4 Discussion

Fungi, like all other organisms, need a continuous iron supply during mycelial growth. Due to the high insolubility of iron at physiological pH, it is sequestered with the aid of special chelating agents, siderophores, as previously discussed. Iron containing proteins and enzymes in micro-organisms initiate and facilitate some very important biological functions e.g. hydroperoxidases like catalase and peroxidase, electron transfer by cytochromes, iron sulphur proteins, nitrogenase, and iron flavoproteins etc (Neilands, 1974). The micro-organisms therefore have to compete with other organisms to acquire iron for such essential enzymes.

In the natural environment it is known that micro-organisms come in contact with other organisms and have to compete for both essential nutrients (eg., carbon and nitrogen), and supplements such as metal ions for growth. Some fungi antagonise other competitive fungi during interaction by production of inhibitory compounds such as soluble metabolites, volatiles, enzymes etc as previously discussed in chapter 3 (sections 1, 2 and 3). However, only recently has the role of iron chelating compounds, in microbial interactions in the rhizosphere been investigated. Plant growth promoting activities of beneficial bacteria, for example ***Pseudomonads***, are enhanced by the production of siderophores. Suppression of disease-causing fungi such as ***Fusarium oxysporum*** or ***Gaeumannomyces graminis*** can be achieved not only by addition of pseudomonads to conducive soils but also by adding their purified siderophores (Kloepper ***et al.***, 1980 a, b; Neilands and Leong, 1986; Schippers ***et al.***, 1987; Sneh ***et al.***, 1984).

Protection of plants against soil-borne fungal diseases has also been achieved with certain fungi. Strains of ***T.harzianum*** and ***T.hamatum*** have been used to control damping off of bean, tomato, and egg-plant seedlings or black-root rot of strawberries (Chet, 1987; Cook and Baker, 1983). Many ***Trichoderma*** have been shown to be mycoparasitic, however production of siderophores and their role in antagonism by these fungi has not been investigated. For growth and survival in a low nutrient, iron limited environment like the rhizosphere or wood, the ability to compete successfully

for iron might play an important role. Another reason to account for the lack of study of siderophores as an antagonistic mechanism against wood decay fungi is that interaction experiments have been undertaken on high nutrient media, like malt extract agar in which the effect of such iron chelating compounds would not be detected. This mechanism however could play a crucial role in the rhizosphere or wood where there is a low availability of metals ions.

Recently Buyer ***et al.*** (1989) devised a new growth media for the study of siderophore-mediated interactions, called the rhizosphere medium (RSM). The inorganic constituents of this medium were designed to mimic the rhizosphere while the organic composition was designed to promote rapid growth and siderophore production. They found that antibiosis experiments were highly reproducible and pseudobactin, a siderophore produced by a ***Pseudomonas putida*** strain, seem to be responsible for antagonising the growth of the take-all fungus ***Gaeumannomyces graminis*** var. ***tritici.*** In order to ensure that the results obtained in artificial media as part of this study would be indicative of natural interactions in wood, studies were carried on a low nutrient media devised, to provide a closer nutritional representation of wood. Neilands (1984 a) has reported that siderophore production is induced when the iron concentration is between 0.1 and 10 μM. The low nutrient media with the lower concentration of iron (0.65 μM) was therefore used in order to study the importance of siderophore competition as an antagonistic mechanism.

The interaction of the selected ten ***Trichoderma*** isolates with the two basidiomycetes in the low nutrient media at the two different iron levels produced some interesting results (table 3.4.3.1). It was clear that the outcome of interactions seen on the LNM were not always reproduced in the LNM+reduced Fe.

The results indicated that although browning at or near the interaction zone indicated in most cases death of the brown rot fungus no browning was required to signify death of the white rot fungus. In most cases interaction/kill with the brown rot fungus showed browning in both media (with the exception of B13 and B15; Figure 3.4.3.1 (c) and (d)) however it was found that the browning was more intense in many interactions on the

reduced iron media. Interactions with the white rot fungus however failed to show any browning on both the media. Other authors have reported (Rayner and Webber, 1984) that interaction between organisms on artificial media, can either lead to deadlock, in which the mycelium of each combatant is unable to enter the other's domain, or replacement where elimination of one by the other results. There may be inhibition of extension by one or both colonies, which may result in pigment production, lysis, and/or major morphological changes. The latter may include production of massed fronts of hyphae, sometimes aggregated into distinctive linear organs or pseudosclerotial plates, which serve to hold or extend domain. Interactive events may often begin before contact, and may indicate the involvement of broad-range antibiotics, generation of an unfavourable pH, or possibly the acquisition or competition for iron between siderophores of the antagonist and target fungi (Rayner and Webber, 1984).

In the case of brown rot fungi, the pigment released during browning may be an indication of the intensity of inhibition as either, 1) a defence mechanism (by formation of pseudosclerotial plates) or 2) as a result of cell death and leakage (senescense). The release of such pigments may be as a result of damage caused by lytic enzyme activity like that discussed in section 3. Chesters and Bull (1963) in their investigation of β-glucanase activation, that was restricted to cellulases and laminarinases showed that in the presence of Fe^{3+} and other metal ions the enzyme activity was significantly increased. They concluded the stimulatory effect of such ions on laminarinase is probably a result of loose binding of the ion to the enzyme and substrate thus inducing configurational and/or energetic modulations which enhanced the rate of hydrolysis. It is possible that the ***Trichoderma*** isolates tested in this study are more efficient at sequestering iron from the medium and that this indirectly increases their lytic enzyme activity and therefore caused increased cell death, as indicated by the more intense browning of the brown rot fungus in the low iron medium. This mechanism is perhaps more significant in the case of the antagonism exhibited by isolates B11 and R10, which produced more intense browning of the ***Neolentinus lepideus*** mycelium in the LNM containing reduced levels of iron.

With white rot fungi, however the browning seen during interaction has been related to the formation of melanin pigments (Collins ***et al.***, 1963), which may be due to cell death and leakage (senescense) or as a defence mechanism. Molecular aspects of cell death has been studied with the filamentous fungus ***Podospora anserina*** (Esser, 1990). In the case of inter-specific interaction between ***Phlebia radiata*** and ***Stereum hirsutum*** (White and Boddy, 1992) and intra-specific antagonistic interactions of ***P.weirii*** (Li, 1981) on agar media pigmented zone lines were noted, which produced stronger phenoloxidase and peroxidase reactions at the interaction zone than the adjacent areas. Browning of fungal tissues resulting from enzymic oxidation of phenolic substrates by phenoloxidases and peroxidases has been commonly observed (Collins ***et al.***, 1963). The initial products of oxidation are usually o-quinones, which being highly unstable, undergo polymerisation to yield dark melanin-like pigments of high molecular mass. The relationship between the presence of melanin, or melanin-like pigments in fungi and resistance to microbial lysis has been demonstrated (Lockwood, 1960; Linderman and Toussoun, 1966; Potgieter and Alexander, 1966, Bloomfiled and Alexander, 1967; Kuo and Alexander, 1967 and Bull, 1970). Bull (1970) reported that melanins can function as a physical barrier, preventing access by cell wall-degrading enzymes of other organisms.

These enzymes, i.e., phenoloxidases and peroxidases have protoporphyrin IX prosthetic groups that have a single ion centres for Cu^{2+} and Fe^{3+} respectively. Therefore absence or unavailability of these ions will result in lack of activity of these enzymes. It is possible that during the interaction between white rot fungi and ***Trichoderma*** species, the ability of the antagonist to sequester the iron by siderophores in the media more efficiently results in 1) lack of growth of the white rot due to iron deficiency and 2) lack of ability to use the peroxidase enzymes to produce melanin or melanin-like pigments that are thought to provide resistance to microbial lysis. However, the phenoloxidases and peroxidases require the presence of phenolic substrates from which the melanin is eventually produced (Chet and Henis, 1969). Though no phenolic compounds were included in the low nutrient media, it is known that micro-organisms can convert aliphatic amino acids, like asparagine, that was added to the medium, to aromatic amino acids like phenylalanine, tyrosine, tryptophan etc. by the sikimic acid pathway and these

may be some of the initial aromatic compounds needed for the synthesis of melanin pigments (Lehninger, 1982).

Chet and Henis (1969) showed that presence of chelators like disodium-EDTA in the growth media prevented melanin-like pigment formation by the sclerotia of the fungus ***Sclerotium rolfsii*** which made them susceptible to laminarinase and chitinase enzymes. It is possible that the chelators affected pigment production by sequestering all the iron, indirectly making the fungal mycelium more susceptible to attack by lytic enzymes. It is interesting to note that interactions in the malt extract agar between ***Trichoderma*** and the ***Trametes versicolor*** (Chapter 2) showed browning at the interaction zones and it can be speculated that this is due to lack of competition between siderophores in the malt medium due to plentiful supply of iron, which allows activity of enzymes involved in pigment production. In the LNM with reduced iron however due to competition by siderophores its availability is limited to the phenoloxidase enzymes indirectly affecting pigment production. Therefore in the former medium there was free available iron for the production of pigments either as a defence mechanism or due to cell leakage after interaction with the antagonist.

This lack of browning after a killing effect is also seen during the interaction with ***N.lepideus***, but only with ***T.pseudokoningii*** isolates B13 and B15. B13 (figure 3.4.3.1 (c)) does not show any browning in interaction with the brown rot fungus in either media type, however B15 (figure 3.4.3.1 (d)) shows browning in the high iron media which is absent in the low iron media. This latter isolate was different from the others in producing an orangey/yellow siderophore colouration in the agar. While white rot fungi produce melanin pigments as a result of phenoloxidase enzyme activity during interaction with other fungi, this enzyme could not be responsible for pigment production in the brown rot fungus, since white rot fungi are unique in production of these extracellular phenoloxidases and peroxidases. This fact is often used as a means of distinguishing the two decay types (Stalpers, 1978; Poppe and Welvaert, 1983). It is possible that the pigments produced by brown rot fungi are biosynthesised by an entirely different pathway, however to date there is very little information published on this area. Brown rot fungi however have been shown to produce phenolate type

siderophores (Jellison *et al.*, 1991), biosynthesised from aromatic precursors, which are generally produced by the sikimic acid pathway of micro-organisms (Young *et al.*, 1967; Lankford, 1973, Robinson *et al.*, 1992). It is possible that these aromatics can also be used for production of some type of pigment as they usually form the precursor substrates for most pigment production.

The growth of the ***Trichoderma*** isolates on the chrome azurol S medium (CAS), and the other two media amended with nutrients of the low nutrient medium (CAS+LNM) and malt extract agar (CAS+MEA), indicated that siderophore production is induced in both CAS and CAS+LNM but not in CAS+MEA, as the iron content in this medium is too high for its induction. However, there was a certain degree of variation in the size of halos and colour in the two media. Schwyn and Neilands (1987) in order to ascertain the behaviour of different types of mutants for siderophore production used the enterobactin of ***Escherichia coli*** and examined the pattern of halo formation on CAS medium. Wild type strains only produce levels of siderophores to satisfy their requirements for the metal, and as a result produces relatively small halos around the colonies when grown on CAS medium. Due to polymerisation of the blue dye, diffusion of siderophores seems to be minimal and the zones are well focussed.

A mutation in the ferric uptake regulation (fur) gene is known to result however in constitutive derepression of the high affinity iron uptake system, resulting in wider halos than the wild type (Hantke, 1981). Other strains containing a mutation in the transport gene (fepB) may deprive itself of iron by its own siderophores. Consequently the synthesis and excretion of enterobactin is fully turned on but the organisms growth is very slow, which results in large halos around small colonies. Other types of mutants are blocked at different points in the siderophore biosynthetic pathway, for example they can be deficient in the production of the intermediate 2,3-dihydroxybenzoic acid, or cannot use such compounds for synthesis of enterobactin (Neilands, 1981 b). These strains do not show any halo, although they are able to grow and must obtain iron via a low affinity pathway. It is possible that similar genetic mutants are found in fungi and this may account for the different types of halos produced by the ***Trichoderma*** isolates in this study. For example with ***T.aureoviride*** isolate B1, no halos were evident in CAS

large halos with very little growth (Figure 3.4.3.2 (b)) and this could be due to a mutation in the fungal siderophore genes that affect iron uptake or transport.

Such variations in the amount of siderophores produced may be very significant for biocontrol of wood decay fungi. For example the mutants that have an ineffective ferric uptake mechanism or a defective transport gene may produce excess siderophores as indicated by their wider halos and slower growth, which may result in binding of the majority of the iron and deprive the wood decay fungi thereby of iron inhibiting their growth and decay activity. If the siderophores produced by the ***Trichoderma*** isolates have a higher or equally good stability constant to those of the basidiomycetes siderophores then, iron will randomly change chelators and remain bound to the one with the higher affinity (Winkelmann, 1973). In general the phenolate type siderophores have a higher affinity than the hydroxamates. It has been shown by TLC and the NMR studies carried out here that ***Trichoderma*** species can also produce phenolate type siderophores, (previously thought to be unique to basidiomycetes) and it is therefore plausible that the chelating ability of the phenolate siderophores which are produced will enable these antagonists to compete more efficiently for the iron (Srinivasan ***et al.***, 1993).

Receptor sites for siderophores on the fungal cell membranes are of equal importance as the concentration of the siderophore produced. Winkelmann (1990) has found that irrespective of the actual mechanism involved in the uptake of iron, specific recognition of the appropriate siderophore by membrane components is a major determinant of iron uptake. The stereochemical structure, i.e., the amino acid backbone and the residues near the metal center, all play an important role in the recognition of the siderophore at the receptor site. It has been noted frequently in bacteria that although they may be deficient in siderophore biosynthesis, they possess multiple siderophore receptors, including receptors for fungal siderophores. Under iron stress for example, ***Escherichia coli*** expresses at least four siderophore receptors Fhu A (ferrichrome), Fep A (Enterobactin), Iut (aerobactin), Fhu E (coprogen) and an additional iron-di-citrate system (Braun and Winkelmann, 1987). A similar situation is found with ***Arthrobacter*** and related organisms where transport of ferrioxamines and ferrichromes is observed

(Huschka *et al.*, 1986). While other microbial species, such as ***Streptomyces pilosus***, use a low specificity, high-affinity transport system that recognises more than one siderophore type. Irrespective of the strategy, such versatility may provide an advantage under Fe-limiting conditions allowing use of siderophores produced by another organism (Crowley *et al.*, 1991).

Thus, iron from fungal siderophores is not only utilised by fungal strains but also by bacteria which do not produce them or which even depend on siderophores as growth factors (Demain and Hendlin, 1959). The fact that prokaryotic organisms are capable of such survival strategies, it cannot be ignored that this might also be applicable to the eukaryotic fungi, that grow in very nutrient limiting environments and may employ similar strategies for iron acquisition. Though there is very little evidence from genetic studies that fungi employ such strategies, the fact that certain fungal siderophore types, eg., ferrichromes, are commonly produced by a wide range of fungi which in addition also produce other types of siderophores, suggests that they may have more than one siderophore receptor. Studies carried out with the fungus ***Neurospora crassa*** showed competition between the ferrichromes and the coprogens, and it was concluded that they may have a common transport mechanism (Huschka *et al.*, 1985). However ***Neurospora crassa*** does not transport ferrirubin and ferrichrome A which do not fit into the receptors of the transport system for that fungus (Winkelmann, 1974). Thus the ability of fungi to possess specific receptors for siderophore uptake may be used as a survival strategy, where they may be able to mimic receptor sites of more than one siderophore or change the same receptor site to allow entry of more than one type of siderophore, eg., ***Penicillium*** spp. (Winkelmann, 1990). If ***Trichoderma*** spp. in the vicinity of wood decay fungi were capable of such strategies then they could deprive the decay fungi of iron that is important for growth.

Such receptor sites in bacteria have been shown to be blocked by an array of lethal agents including antibiotics, bacteriophages, and bacteriocins (Neilands, 1979). It is possible that even fungal siderophores or other metabolites produced by them can also affect siderophore receptors of their target pathogen and deny them of iron. A few non-selective antibiotics such as stemphyloxins, syringomycin, marasmins, furasic acid, and

ascorbitine, exhibit chelating properties (Barash and Manulis, 1986). These compounds share some properties with siderophores, namely, regulation by iron and specific binding of ferric ion. However, they differ from siderophores in their dependence on low iron concentration for biosynthesis and a distinctive, lower binding constant for ferric ion (Manulis ***et al.***, 1987 a). Such phytotoxic chelates produced against plants by fungi could also be produced against other fungi as a defence mechanism, and it is possible that the killing effect seen in iron sufficient media (LNM) may be due to such antibiotics that have a dual role of being toxic and capable of iron assimilation at higher iron concentrations than that of siderophores. This will enable the fungus to not only sequester iron in the media more efficiently, but also to have an antibiotic effect that will retard growth and result in death of any competitors.

Triacetylfusigen, a siderophore produced by many ***Aspergillus*** and ***Penicillium*** species was also noted by Anke (1977) to possess an antibiotic effect. Though several trihydroxamates excreted by actinomycetes e.g. sideromycins and nocardamine (desferroxamine E) were known for their antibiotic activity (Stahl, 1967, as cited in Anke, 1977), Anke (1977) was the first to show the antimicrobial effect of triacetylfusigen. Anke (1977) found that the antibiotic activity was dependent on the composition of medium under test. On addition of the siderophore to the medium only bacteria were found to be sensitive. When grown on a minimal medium however most organisms were found to be sensitive. Even yeasts became sensitive to some extent when grown on minimal media. This antibiotic activity could be eliminated on addition of iron (III).

The production of siderophores by ***Trichoderma*** with higher affinity than that of the basidiomycetes siderophores, apart from playing an important role in the inhibition of growth may also affect the enzymic degradation of wood by the basidiomycetes. Many mechanisms involved in wood degradation may be affected by the absence of iron.

A currently held hypothesis considers that as enzymes are unable to penetrate into the wood cell wall and cause degradation, because the micropores (sub-microscopic size holes, known to exist in the wood cell wall) are not large enough to allow enzyme

penetration, a separate non-enzymatic system involving metal ions (iron or perhaps manganese or copper), and a radical generating system may be involved (Highley, 1980; Koenigs, 1974; Murmanis ***et al.***, 1988 a). This system, is thought to operate as an initial decay step within the cell wall, opening up the pores to allow the enzymes to penetrate the microstructure. Degradation patterns differ between different types of decay fungi; however, non-enzymatic degradation systems have been proposed for both of the two basic types of decay; brown-rot and white-rot (Srebotnik and Messner, 1988; Srebotnik ***et al.***, 1988).

Brown-rot decay fungi primarily degrade cellulose and hemicellulose, often degrading cellulose in a process similar to acid hydrolysis, at a rate in excess of what they can utilise. An enzyme capable of duplicating these effects in wood has never been isolated from a brown-rot fungus and cellulase enzymes thus far isolated are too big to diffuse freely into the wood microstructure. Brown-rots produce a constitutive enzyme complex with endo-1,4-β-D-glucanase activity, but many brown-rot fungi apparently lack exo-β-1,4-glucan cellobiohydrolase activity (Kirk and Cowling, 1984). Involvement of a non-protein hydrogen peroxide/iron system via an oxidative mechanism has been suggested as an explanation for the ability of brown-rot fungi to vigorously degrade wood cellulose in the absence of detectable exo-1,4-β-glucanase activity (Highley, 1980; Koenigs, 1974).

White-rot fungi produce endo-1,4-β-glucanases, exo-1,4-β-glucanases and β-glucosidases which act synergistically to degrade cellulose (Shoemaker, 1985), and a non-enzymatic depolymerisation agent also appears to play a role in white-rot degradation. Further insight into cellulose and hemicellulose degradation by both brown and white fungi can be obtained from numerous recent studies on the filamentous fungi (Enari, 1985; Shoemaker, 1985; Warren, 1986; Knowles ***et al.***, 1987).

Lignin can be completely metabolised to CO_2 by white-rotting fungi. Several enzymes are involved in lignin breakdown including lignin peroxidase, manganese peroxidase, laccases, and oxidase. Lignin peroxidase catalyses the H_2O_2 dependent oxidation of lignin. Substrates can be oxidised by 1 or 2 electrons. The main isozyme form of the

enzyme is a glycoprotein with a molecular weight of 41,000 daltons. The enzyme is of broad specificity, and is capable of causing the partial depolymerisation of lignin. Since the isolation of the first ligninase, other enzymes capable of participating in lignin break-down have been isolated with specific lignin peroxidases isolated in multiple forms (Kirk ***et al.***, 1986). All ligninases examined have a single iron protoporphyrin IX prosthetic group. Another ligninase, MnII-dependent peroxidase, has also been isolated (Glenn and Gold, 1985). This enzyme is a 46,000 MW glycoprotein with a heme prosthetic group and is dependent on both H_2O_2 and MnII for activity. Unlike other extracellular ligninases the MnII peroxidase appears to be associated closely with the fungal hyphae (Paszczynski ***et al.***, 1986).

Iron and other metals such as manganese, play a role in biological degradation as essential elements for fungal metabolism and growth. And it is clear from examining the mechanisms involved in wood degradation that in addition, iron also plays an important role in biodegradation of wood as : 1) a component of the extracellular heme enzymes involved in white-rot degradation (Paszczynski ***et al.***, 1988; Tien and Kirk, 1983); and 2) possibly in brown-rot organisms in a non-enzymatic iron/hydrogen peroxide catalysis of cellulose degradation (Highley, 1980; Koenings, 1974; Murmanis ***et al.***, 1988 a).

To solubilise and sequester ferric iron basidiomycete fungi also produce siderophores like many other fungi. The ability of selected decay fungi to produce siderophores has been demonstrated (Fekete ***et al.***, 1989) using a modification of the Chrome Azurol-S assay (Schwyn and Neilands, 1987) and a rapid paper electrophoresis assay (Fekete ***et al.***, 1983). The compounds isolated from the decay fungi were found to be phenolate in character. Evidence of biological chelators being produced was obtained for ten degradative fungi with some organisms, such as ***Trametes versicolor***, showing evidence of producing up to three forms of iron chelating metabolites (Fekete ***et al.***, 1983 and 1989).

These authors have isolated siderophores from ***Gloeophyllum trabeum*** (brown rot) and ***Trametes versicolor*** (white rot) and have demonstrated the iron-repressibility of these chelators. Experiments by Dolphin, 1986, Dolphin ***et al.***, 1987, and Paszczynski ***et al.***,

1988, which demonstrated activity of different porphyrins and non-biological chelators on lignin, suggest the possibility of direct involvement of siderophores in the process of wood degradation.

The mechanism behind enzyme penetration into the wood cell wall is still unclear and it is possible that enzymes penetrate the wood cell wall only in late stages of cell wall attack. However, there is some evidence to suggest that large molecular weight glycoproteins such as degradative enzymes could move into existing microvoids in some plant tissue cell walls prior to extensive cell wall attack (Jellison and Goodell, 1988). An alternative hypothesis has been put forward by Jellison ***et al.***, 1990 for the mechanism of degradative fungal action in wood. Jellison ***et al.*** (1990) also suggested that the action of metal-chelating siderophores produced by decay fungi may function to chemically modify unspecified groups within wood similar to the function of synthetic chelator compounds reported previously by other researchers. At this point, it is unknown how closely siderophores may associate with enzymes, or if they can function in wood to penetrate into the wood cell wall, potentially "opening" the wood structure for further enzymatic attack.

Apart from having an important role in the biodegradation of wood iron may also be involved indirectly in the production of the hyphal sheaths by the wood decay fungi that are thought to carry the enzymes to sites of degradation. The involvement of an extracellular glycan sheath in wood degradation has been shown for ***Phanerochaete chrysosporium*** (white-rot) (Ruel and Joseleau, 1991) and has been investigated for the brown-rot fungi (Green ***et al.***, 1991 a, b; Green ***et al.***, 1989 b). Iron regulation of capsular polysaccharides has been shown for ***Azotobacter chroococcum*** B-8 (Ferrala ***et al.***, 1986; Fekete ***et al.***, 1987). If the sheath thickness of wood decay fungi is also dependent on iron concentration, then it may be reasonable to suggest that the sequestering of iron by siderophores would influence glycan sheath production. Since these glycan sheaths apart from being carriers for degradative enzymes may also be involved in the protection of mycelium from attack by soluble and volatile antibiotics, as discussed earlier in Chapter 3, non-availability of the iron needed for sheath

production due to competition by siderophores may facilitate antagonism by the ***Trichoderma***.

Some indirect evidence exists on the effect of non-biological chelators and some iron-binding siderophores on brown-rot decay. Viikari and Ritschkoff, (1992) have shown that brown-rot decay was prevented by chelating the endogenous metals in wood. The fungal growth and decaying ability of selected basidiomycetes were significantly decreased by the chelating treatments of the solid wood-based culture medium and pine wood pieces, respectively. Siderophores of antagonists like ***Trichoderma*** may also limit decay in wood by a similar mechanism.

It is clear however that siderophores are likely to have an important role to play by sequestering iron for the purpose of enzymatic activity or non-enzymatic degradation of wood. If the presence of another organism such as ***Trichoderma*** in the vicinity of the basidiomycetes competes for the limited available iron that is needed for the functions of degradation it can indirectly affect the growth of the organism. The fact that ***Trichoderma*** have also been shown to produce the phenolate type siderophores (Srinivasan ***et al.***, 1993) like basidiomycetes, that are more effective iron chelators than hydroxamates, strengthens the arguement for siderophore competition in biocontrol of wood decay fungi.

To establish their role and inorder to totally understand their potential for competition with basidiomycete siderophores it is important to estimate the rate and type of siderophore production by ***Trichoderma*** spp. Radiolabelled siderophores may be used to determine the formation constant of purified siderophores. Rate of production of the siderophores however will be very much dependent on the environment of growth whether in artificial media or in wood.

If it is possible to retard growth of basidiomycetes and eliminate wood degradation by depriving these fungi of iron, therefore this might be a very important biotreatment method to protect wood and may allow the development of a specific, efficient and non-toxic wood preservative system.

Chapter 4

Basidiospores

Chapter 4

Inhibition of Basidiospore Germination

4.1 Introduction

Basidiomycetes represent the largest and most highly evolved class of fungi with approximately 15,590 species. Complex life cycles including the production of many different types of spores are common in basidiomycetes. Spore types include basidiospores, pycniospores, aeciospores, uredospores, teliospores, conidia, and chlamydospores (Weber and Hess, 1976). In comparison to the morphological diversity of other spores, basidiospores are rather more uniform. Typically, they are unicellular, but transversely septate spores are found in certain groups. In shape, they vary from globose, sausage-shaped, fusoid and almond-shaped, and may contain ridges or folds. The colour of basidiospores is an important criterion of classification. They may be colourless, white, cream, yellowish, brown, pink, purple or black (Webster, 1980).

Most screening of potential biocontrol agents of wood decay fungi has previously been undertaken against basidiomycete mycelium. Similarly experiments designed to evaluate the mechanisms involved in antagonism (soluble metabolites, volatile antibiotics, lytic enzymes and siderophores) between biological control agents and target fungi have always been carried out using mycelial inocula. Basidiospores are a primary source of infection leading to decay of wood exposed above ground and are the pathogenic form of the target fungus that is first in contact with the biocontrol antagonist. However most of the experiments undertaken as part of this study to evaluate the potential of biocontrol agents has involved the use of mycelial inocula rather than basidiospores. Studies on the prevention of basidiospore germination require a large supply of viable basidiospores. Such basidiospores are difficult to obtain naturally and asceptically at an appropriate time for experimentation.

Previous research on ***in vitro*** carpogenesis and basidiosporogenesis of various wood decay basidiomycetes has demonstrated the variability of the process (Morton and French, 1966; Schmidt and French, 1977; Bjurman, 1984, 1987, 1988; Croan and

Highley, 1992). Induction of cultures to produce carpophores and basidiospores ***in vitro*** is a difficult task. Consequently, little information is available on mass production of basidiospores ***in vitro*** and factors affecting it, most researchers therefore have studied the effect of inhibition of the mycelial growth of wood decay fungi rather than basidiospore germination.

Todate no work has been published with regard to the antagonistic effect of ***Trichoderma*** spp. against basidiospores. The work reported in this chapter examines the inhibitory effect of soluble metabolites and volatile antibiotics of ***Trichoderma*** spp. against wood decay basidiospores.

Materials and Methods

The ***Trichoderma*** isolates used as antagonists are the ten strains selected previously on the basis of interaction studies in chapter 2. The basidiospores used as target pathogens consisted of, the brown rot fungus, ***Neolentinus lepideus*** (MAD-534) and two white rot fungi, ***Trametes versicolor*** (MAD-697) and ***Phanerochaete chrysosporium*** (Karsten)(BKM F1767). Also used were the spores of the sap-stain fungus, ***Aureobasidium pullulans*** (deBary) Arnand (MDX 18), and conidia of two mould fungi, ***Aspergillus niger*** van Trieghens and an unknown ***Trichoderma*** spp. All spores used were kindly provided by Suki Croan, Forest Products Laboratory, Madison, Wisconsin.

4.2 Viability Test of Basidiospores

Once spores had been harvested onto grease proof papers, they were freeze dried at a low temperature for 2 to 3 hours and sealed in air tight petri dishes and stored at 2 °C. The viability of basidiospores were tested according to the following procedure. Small pieces of the paper with the basidiospores were cut asceptically and suspended in 0.005% tween (sterile) by vortexing vigorously for a few seconds, the paper was then immediately removed asceptically (the tween prevents the basidiospores from clumping together). This suspension was then treated as the stock solution of basidiospores. To determine the number of basidiospores in the stock solution a total count was done with the aid of a haemocytometer (1:1 volume of trypan blue dye (%) with the stock solution made counting easier). Plates of MEA and LNM were inoculated over a diameter of 3

cm at the centres of the plate with either the stock solution of the basidiospores or a sample diluted in sterile water. Dilutions were made so that the total count of basidiospores plated ranged between 200 to 400 spores / 3 cm (diameter) area for ease of viability counting. These plates were then incubated at 25 °C overnight in the dark. Viability counts were done by noting the number of germinated and non-germinated basidiospores after the incubation period with the aid of a microscope (x 100 magnification). All basidiospores used in the tests that follow had viabilities around 80 %.

Effect of selected modes of antagonism against basidiospores

The antagonistic effect of the ten selected ***Trichoderma*** isolates with regard to production of inhibitory soluble metabolites and volatile antibiotics against the basidiospores of the brown rot fungus ***N.lepideus*** (MAD 534) and the white rot fungus ***T.versicolor*** (MAD 697) was tested.

4.2.1 Inhibition of Basidiospores by Soluble Metabolites

Cores of ***Trichoderma*** isolates (0.6 cm in diameter) were inoculated centrally onto plates of each of the two media by (3 % ME (Oxoid L39), 2% Nobles agar (Difco B142) and LNM autoclaved at 121 °C for 15 min) and incubated for 4 days at 22 or 25 °C. These plates were then covered with a sterilised circular piece of cellophane membrane (8.4 cm diameter discs autoclaved at 121 °C for 15 min in glass petri plates) to exactly cover the agar surface. These plates were then inoculated centrally with 1 ml of either ***N.lepideus*** or ***T.versicolor*** basidiospores (200, 000 spores/ml) and the spores spread evenly over the cellophane with a sterile bent glass rod. Four replicate plates were set up per treatment. The control plates consisted of plates of both media types (uninoculated with ***Trichoderma***) overlayed with cellophane and inoculated with either type of basidiospores. The plates were checked on a daily basis for germination of basidiospores with the aid of a binocular microscope (X 40 magnification) for a period of 10 days.

4.2.2 Inhibition of Basidiospores by Volatile Antibiotics

Agar plates of each medium (3% ME, 2% Nobles agar and LNM) were inoculated centrally (0.6 cm diameter core) with each of the ***Trichoderma*** isolates and the two basidiospores spread on either media types (1 ml containing approximately 200, 000 spores). Plates containing basidiospore inocula were covered with a semipermeable polythene membrane and inverted over the plates containing individual ***Trichoderma*** isolates and incubated at 22 or 25 °C. Four replicates were set up per treatment. Appropriate controls for each medium consisted of plates of either basidiospores on the respective media superimposed over uninoculated plates of the same media. The plates were checked daily with the aid of a binocular microscope (X40 magnification) over a period of 10 days.

4.2.3 Antifungal Activities of Soluble Metabolites using Plate Bioassays

The ten ***Trichoderma*** isolates were inoculated (one 0.6 cm diameter plug per flask) into 500 ml of each of the two media types (3 % ME and LNM) and grown for 14 days at 22 or 25 °C. After the incubation period the fungal mycelium was removed by filtration and the culture filtrate sterilised by passing through a 0.45 µm sterile membrane filter (Whatman) and then passed through a membrane filter with a 1000 dalton molecular weight cut off to remove any enzymes in the filtrate. The filtrates were then lyophilised to concentrate the soluble metabolites. The lyophilised filtrates were resuspended in 10 ml of sterile distilled water to achieve a 50 fold concentration of the antifungal agent.

To test the activity of the inhibitory substances released by the ***Trichoderma*** isolates a plate bioassay (Croan and Highley, 1991) was carried out as follows. Separate 1 ml spore suspensions of either ***N. lepideus***, ***T. versicolor***, ***P. chrysosporium***, ***A. pullulans***, ***A. niger*** or the unidentified ***Trichoderma*** isolate (200, 000/ml for conidia and 400, 000/ml for basidiospores) were spread plated onto 2% ME, 2% Nobles agar plates. Six 6 mm diameter wells placed equidistant from one another were cut with a cork borer and 100 µl of the concentrated filtrates of all the ***Trichoderma*** isolates grown on each of the two media were pipetted into the wells. Also added in one well was the crude metabolite of ***Streptomyces rimosus*** Sobin (10 µl) for comparison (Crude metabolite preparation of

St.rimosus was obtained also from Suki Croan, as described in Croan and Highley, 1992). The clear zone-diameter around the wells were measured after incubation at 27 °C for 3 days.

4.3 Results

4.3.1 Effect of soluble metabolites on basidiospores

Figures 4.3.1 (a) and (b) show the inhibition of germination caused by soluble metabolites of the ***Trichoderma*** isolate ***T.harzianum*** (B11) against both basidiospores on malt extract and low nutrient media. Results presented in table 4.3 indicate that inhibition of germination was 100% by all ***Trichoderma*** isolates against both ***N.lepideus*** and ***T.versicolor*** basidiospores on both media types, with the exception of R7 and R10 (***T.viride*** isolates), which showed lesser inhibition of germination of the ***T.versicolor*** basidiospores on the low nutrient media.

Lack of germination inhibition of ***T.versicolor*** basidiospores, by R7 and R10 (***T.viride***) isolates in the low nutrient media, once again stresses the importance of media selection for such studies. As soluble metabolites from these isolates had however shown complete inhibition of growth of the mycelial inoculum of ***T.versicolor*** in the same low nutrient media, it is possible that some other antagonistic mechanism than soluble metabolite production is involved in inhibition of germination or that different metabolites may be specific in inhibiting the mycelial form of the wood decay fungus.

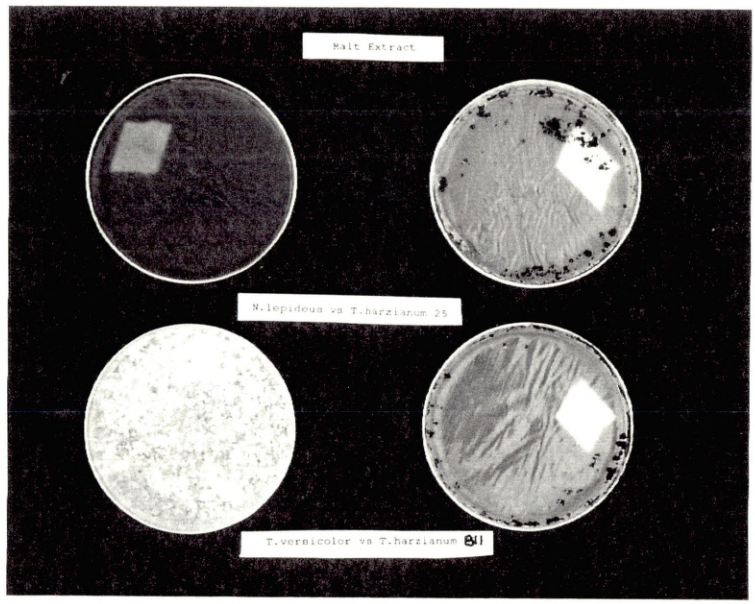


Figure 4.3.1 (a) - Inhibition of *N.lepideus* and *T.versicolor* basidiospore germination by soluble metabolites of *T.harzianum* (B11) grown on malt extract agar. Plates on the left are the controls.

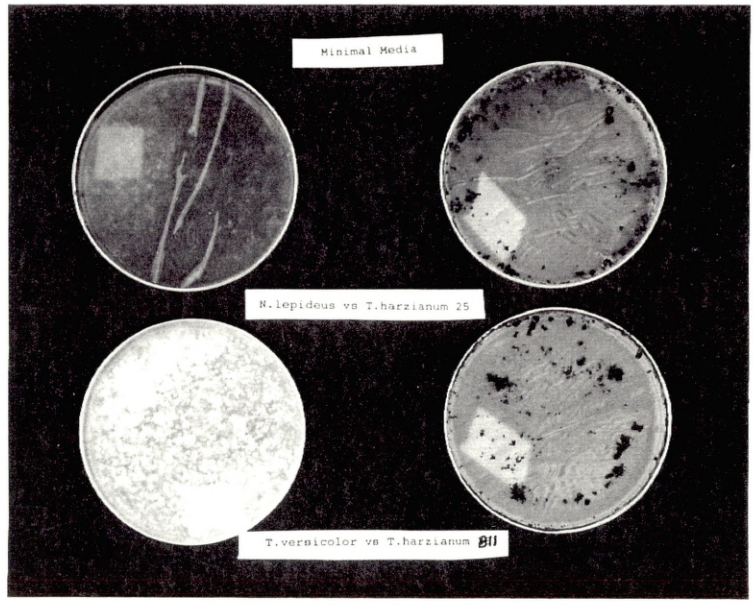


Figure 4.3.1 (b) - Inhibition of *N.lepideus* and *T.versicolor* basidiospore germination by soluble metabolites of *T.harzianum* (B11) grown on low nutrient media. Plates on the left are the controls.

Among the ten ***Trichoderma*** isolates chosen for further work on the basis of preliminary interaction studies, three isolates B1 (***T.aureoviride***, B11 (***T.harzianum***) and R2 (***T.viride***), that did not show complete inhibition of one or both basidiomycetes on the two media types, were included as control organisms. Interestingly, although these three isolates previously did not inhibit growth of basidiomycetes, they inhibited germination of the basidiospores. It is also evident from previous results on % inhibition of mycelial growth of the basidiomycetes by soluble metabolites (Chapter 3, section 1), that the inhibitory effect of soluble metabolites exhibited by these isolates was either low or nil. It is possible therefore that the soluble metabolites produced by these isolates are directed specifically against basidiospores.

4.3.2 Effect of volatile antibiotics on basidiospores

It is clear from the results presented in table 4.3 that none of the ***Trichoderma*** isolates produce volatiles that are inhibitory to basidiospores on either media type. From previous experimentation (Chapter 3, section 2) it is known that the mycelial form of the same basidiomycetes are inhibited by volatiles from these ***Trichoderma*** isolates. This result therefore indicates that the volatiles produced by the ***Trichoderma*** isolates are directed specifically against the mycelial form of basidiomycetes.

Trichoderma species	Malt extract agar				Low nutrient media			
	N.lepideus		T.versicolor		N.lepideus		T.versicolor	
	Sol	Vol	Sol	Vol	Sol	Vol	Sol	Vol
B1	-	++	-	+++	-	++	-	+++
B5	-	++	-	+++	-	++	-	+++
B11	-	++	-	+++	-	++	-	+++
B13	-	++	-	+++	-	++	-	+++
B14	-	++	-	+++	-	++	-	+++
B15	-	++	-	+++	-	++	-	+++
B16	-	++	-	+++	-	++	-	+++
R2	-	++	-	+++	-	++	-	+++
R7	-	++	-	+++	-	++	++	+++
R10	-	++	-	+++	-	++	++	+++
Ctrl	++	++	+++	+++	++	++	+++	+++

Table 4.3 - Inhibition of basidiospore germination by soluble metabolites (Sol) and volatiles (Vol).

Key - (-) indicates no spore germination and (+..+++) indicates increasing degree of basidiospore germination; Ctrl - Control plates. ***Trichoderma*** isolates - B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - Unidentified ***Trichoderma*** isolate.

4.3.3 Antifungal effects of soluble metabolites in a plate bioasssay

The effects of soluble metabolites on spore germination in the plate bioassay are shown in table 4.3.3. A total of 6 types of fungal spores were used for this study. ***Aspergillus niger***, ***Trichoderma*** and ***Aureobasidium pullulans*** were used as test organisms, as they are primary colonisers and may therefore be likely to be more sensitive to metabolites which inhibit spore germination. However none of the plate bioassays with these three organisms showed any zones of spore germination inhibition with any of the filtrates of ***Trichoderma*** isolates. The three basidiospores used consisted of two white rot fungi, ***T.versicolor*** and ***P.chrysosporium***, and a brown rot fungus ***N.lepideus***.

Filtrates of B1 (***T.aureoviride***) and R2 (***T.viride***) grown on malt extract produced zone diameters of 2.5 and 1.8 cm respectively against ***T.versicolor***. However more ***Trichoderma*** filtrates obtained after growth in the low nutrient media showed inhibition of germination of the ***T.versicolor*** basidiospores. These include ***T.viride*** isolates, B5-1.3 cm, R2-2.5 cm and R7-1.8 cm, and ***T.pseudokoningii*** isolates, B13-1.3 cm, B14-1.3 cm. The zones were noted after four days of growth. It is of interest that the inhibition zone observed with R2 has increased from 1.8 cm to 2.5 cm in diameter where the latter filtrate was obtained from LNM, that is more representative of ***in situ*** nutrients. After incubation for an additional 6 days however, the zones were no longer clear and a few basidiospores could be seen germinating near the wells. Crude metabolite harvests of ***Streptomyces rimosus*** were used for comparison and exhibited an inhibition zone of 4.2 cm which was maintained throughout the 10 days of study.

None of the ***Trichoderma*** filtrates inhibited germination of ***P.chrysosporium*** basidiospores indicating that the metabolites produced by the ***Trichoderma*** isolates are more specific to ***T.versicolor*** even though both organisms tested are white rot fungi. Results could not be obtained from the tests performed with the basidiospores of ***N.lepideus*** due to poor spore viability of lyophilised stock cultures.

Trichoderma species	Basidiospore type					
	T.versicolor		P.chrysosporium		N.lepideus	
	MEB	LNM	MEB	LNM	MEB	LNM
B1	2.5	0	0	0	NT	NT
B5	0	1.3	0	0	NT	NT
B11	0	0	0	0	NT	NT
B13	0	1.3	0	0	NT	NT
B14	0	1.3	0	0	NT	NT
B15	0	0	0	0	NT	NT
B16	0	0	0	0	NT	NT
R2	1.8	2.5	0	0	NT	NT
R7	0	1.8	0	0	NT	NT
R10	0	0	0	0	NT	NT

Table 4.3.3 - Basidiospore germination inhibition zones (diameter in cm) by ***Trichoderma*** filtrates after growth in malt extract broth and low nutrient media. ***Trichoderma*** isolates tested - B1 - ***T.aureoviride***; B5, R2, R7 and R10 - ***T.viride***; B11 - ***T.harzianum***; B13, B14 and B15 - ***T.pseudokoningii***; B16 - Unidentified ***Trichoderma***. NT - not tested.

4.4 Discussion

It is evident from the results presented here and in Srinivasan ***et al.*** (1993 a) that soluble metabolites show differing specificities during inhibition of basidiospores, and mycelial inoculum of basidiomycetes as indicated in (Chapter 3; section 1). It is also evident that the volatiles produced by the ***Trichoderma*** isolates have little or no inhibitory effect on basidiospore germination.

Target specificity seems to play an important role in the antagonism of basidiospore germination by soluble metabolites of ***Trichoderma*** spp. This is indicated by the results, of isolates (R7 and R10, ***T.viride***) which specifically inhibit germination of brown rot rather than the white rot basidiospores. These results confirm conclusions made in previous studies (Srinivasan ***et al.***, 1992 a, b) about target specificity of these antagonists against mycelial inocula of basidiomycetes, where inhibition of the brown rot fungus ***N.lepideus*** was more common than that of the white rot basidiomycete ***T.versicolor***. Highley and Ricard (1988) have reported such target specificity while studying the antagonistic potential of ***Trichoderma*** spp. and ***Gliocladium virens*** against several white rot and brown rot basidiomycetes. These authors reported that ***Trichoderma*** spp. generally prevented or reduced decay by the brown rot fungi except ***Gloeophyllum trabeum***, but were also generally ineffective against the white rot fungi. Such trends seem also to be prevalent with results obtained on inhibition of brown rot and white rot fungal basidiospores. Results of the plate bioassay indicate that target specificity may also occur within the white rot fungi, as no inhibition of the ***P.chrysosporium*** was detected by any of the ***Trichoderma*** filtrates even though germination of basidiospores of ***T.versicolor*** were inhibited by the soluble metabolites of some ***Trichoderma*** isolates.

Inhibition of both basidiomycetes was 100% by almost all the ***Trichoderma*** isolates in the studies when the agar plates with the antagonists were overlayed with cellophane and then seeded with the basidiospores. However this inhibitory effect was reduced to a select few when the concentrated filtrates of the antagonists grown on the two media types (MEB and LNM) were tested in the plate bioassay. It is possible that in the

cellophane test the effect of more than one antagonistic mechanism was responsible eg., lytic enzymes. This mechanism is not present in the plate bioassay as the filtrates were passed through a membrane filter to remove larger proteins such as enzymes.

Also since the clearing zone produced by some filtrates was lost after a certain time period in the plate bioassay, unlike the test filtrate of ***St.rimosus***, it may be concluded that the ***Trichoderma*** filtrates only have a fungistatic stalling effect on the basidiospores and this effect is reduced with time perhaps due to the metabolite becoming exhausted or losing its activity. It may be that the presence of the antagonist is necessary to enforce a constant inhibitory effect to stop germination of the basidiospores and this would account for the total inhibition seen in the cellophane overlay experiment.

Nutrient composition of test media for such studies has already been shown by many to play an important role in determining the outcome of antagonistic responses (Srinivasan ***et al.***, 1992 a,b; Dwivedi and Sharma, 1989; Park ***et al.***, 1991; Carter and Lynch, 1991; Danielson and Davey, 1973 a and b). This is again evident here with the results of the bioassay where more inhibition zones were seen with filtrates obtained after growth in the low nutrient media than in malt extract broth. Perhaps these ***Trichoderma*** isolates that can exhibit an inhibitory effect growing in the low nutrient media may have a greater potential in inhibiting basidiospore germination in wood and it is possible that metabolites which are specific against basidiospores can only be produced in the former media that was devised to be a closer representation of wood.

The results of the study carried out above clearly indicate that there exists a marked difference between the antagonistic effect of ***Trichoderma*** spp. exhibited against mycelial inoculum of basidiomycetes and basidiospores. Since basidiospores are the primary source of decay it is important to direct wood protectants against both mycelium and basidiospores. Spores of decay fungi are also known to be more sensitive to certain wood preservatives than established mycelium (Morton and French, 1966; Schmidt and French, 1979).

Though very little research has been carried out on the inhibition of germination of basidiospores by biocontrol agents such as ***Trichoderma*** and their antagonistic compounds, some researchers have examined the effect of certain pesticides and wood extracts that have a higher target specificity like the soluble metabolites of ***Trichoderma*** spp. Schmidt (1986) examined the effect of the chitin synthesis inhibitor polyoxin D, on basidiospore germination. Though the concentration of plyoxin D required to inhibit germination or cause abnormalities differed between the basidiospores tested, some degree of inhibition of all species tested was produced. Scarlette (1990) noted that certain water soluble and organic solvent soluble extracts from pine wood can affect spore germination. It is known from the results presented in this chapter that soluble metabolites of some ***Trichoderma*** spp. can have a fungistatic and may be in higher concentrations a fungicidal effect over basidiospore germination. Characterisation of these metabolites involved in specific inhibition of basidiospores and study of there mechanism of action will therefore, enable development of more specific bioprotectants.

Chapter 5

Wood Decay Experiment

Chapter 5

Wood decay Experiments

5.1 Introduction

The concept of using biological control agents for the protection of wood and wood products has become an attractive alternative to the use of environmentally unsafe toxic preservatives. Evaluation of the potential of biocontrol agents such as ***Trichoderma***, ***Gliocladium*** and ***Scytalidium*** against wood decay basidiomycetes have been studied by many researchers by both field tests and ***in vitro*** soil block studies (see Chapter 1).

The use of biological agents to control decay of creosoted distribution poles using ***Trichoderma*** and ***Scytalidium*** species has been reported by a number of authors (Ricard ***et al.***, 1969; Ricard, 1976; Bruce, 1983; Morris, 1983; Bruce and King, 1986 a, b and Bruce ***et al.***, 1990). These field studies, have shown variable, and limited success compared with favourable laboratory observations of antagonism between ***Trichoderma***, ***Scytalidium*** and some wood decay organisms commonly isolated from creosoted wood poles.

Highley and Ricard (1988) showed that wood blocks treated with ***Trichoderma*** species could be protected from attack by selected brown rot fungi. Bruce and King (1983) found that wood blocks could be protected from ***N.lepideus*** by ***Trichoderma*** spp. even after killing the ***Trichoderma*** and leaching the wood. Morris ***et al.*** (1986) confirmed this residual protection against ***N.lepideus*** but found that the decay resistance was lost after extended leaching. Morrell and Sexton (1988), however, found only limited success by ***Trichoderma*** in arresting the development of decay fungi in wood blocks. More recently, Freitag and Morrell (1990), evaluated the biocontrol potential of selected microfungi against some basidiomycete decay fungi using a small scale wood sandwich test. They tested the potential of four microfungi for biocontrol of four basidiomycete decay fungi and used the small scale wood sandwich test as well as the more commonly used wood block test. They noted that the smaller scale test with wafers produced

similar results to that of the wood block test in only 6 weeks with respect to the longer 12 week incubation with the latter test.

The variability in results obtained by researchers could be due to a number of reasons. However, one important factor which restricts interpretation of results of wood block experiments is the lack of knowledge with regard to the antagonistic mechanisms which biocontrol agents such as ***Trichoderma*** exhibit in the natural substrate i.e., wood. In order to achieve a high level of consistent field performance by the bioprotectant, more information on its mechanism of antagonism in wood against basidiomycetes needs to be established.

Though many researchers have evaluated the potential of ***Trichoderma*** in protecting wood and also shown the different antagonistic mechanisms that inhibit growth of wood decay basidiomycetes in ***in vitro*** tests by agar plate methods, few have attempted to elucidate which mechanism or mechanisms are important in wood. In this section detailed statistical analysis of wood block weight loss data was undertaken with a view to identify the importance of individual antagonistic mechanisms of ***Trichoderma*** spp. that enable them to exhibit inhibiton against the decay fungi in wood.

5.2 Material and Methods

5.2.1 Wood Block Decay Tests

The method used for testing the biocontrol potential of the ***Trichoderma*** isolates was a modification of the American Standard Test Method (ASTM) for wood preservatives by laboratory soilblock cultures ASTM Designation D 1413 - 76 (Reapproved 1986).

Wide neck soil bottles (250 ml) (Glaserwerk Wertheim, Germany) were filled with 140g of soil set at a moisture content of 130% of the water holding capacity of the soil (see Appendix III). Feeder strips were cut from the sapwood of Scots Pine (***Pinus sylvestris***) and were approximately 0.3 x 3 x 1.5 cm with the grain of the wood parallel to either of the long dimensions. These were then placed on the soil (two per bottle) and the bottles steam sterilised at 15 psi for 30 min. After sufficient cooling of the culture bottles, the

feeder strips were inoculated with a 1 cm (diameter) colonised agar section of each of the ten chosen ***Trichoderma*** isolates (two bottles/isolate). After one week incubation at either 22 or 25 °C and after the ***Trichoderma*** showed good colonisation of the soil and feeder strips, conditioned wood blocks were placed on the feeder strips (2 blocks/bottle, i.e. 4 blocks/organism) and incubated at the appropriate temperatures for a period of 4 weeks. The test blocks 2cm x 2cm x 2cm were cut from Scots pine sapwood with a density of 5 to 4 rings/cm, free of knots and resins, and showing no visible evidence of infection by mould, stain, or wood-destroying fungi. The dry weight of the pre-labelled blocks were determined by placing in an oven at 103 °C for 3 hrs. The blocks were then conditioned in a 25 °C constant temperature room at 70 % humidity for two days, to allow some moisture into the blocks prior to steam sterilised at 100 °C for 20 min before use in the test. After exposure to ***Trichoderma*** isolates blocks were then transferred into another set of soil bottles in which the feeder strips had been precolonised by either of the two basidiomycetes ***N.lepideus*** FPRL 7F or ***T.versicolor*** MAD 697 and incubated for a further 16 weeks at 25 °C.

A second set of blocks pretreated with ***Trichoderma*** isolates as before (4 blocks/isolate) were steam sterilised before placing in the soil bottles with basidiomycetes. Controls included blocks that were pretreated with ***Trichoderma*** (4 blocks/isolate) and not subsequently exposed to basidiomycetes to establish the weight loss of the wood blocks after pretreatment, and others that were not pretreated with ***Trichoderma*** but decayed for 16 weeks by basidiomycetes in soil bottles (12 blocks/basidiomycete treatment).

After incubation wood blocks, whether tests or controls, were removed from jars, their surfaces cleaned by gently brushing off the mycelium and weighed for moisture content and then dried at 103 °C for 3 hrs to obtain their dry weight. They were then transferred into a desiccator and their weight measured to determine weight loss due to the various treatments. The weight loss of the blocks before and after treatment was calculated as follows :

$$\% \text{ weight loss} = 100 \times (T_1 - T_2) / T_1$$

where T_1 is the oven dry weight of the wood blocks before any treatment and T_2 is the dry weight either after the 4 week incubation with the ***Trichoderma*** or the 16 week incubation with the basidiomycetes.

5.2.2 Statistical correlation of wood decay experiments with results of individual antagonistic mechanisms - Stepwise Regression Analysis

Stepwise regression analysis (Draper and Smith, 1981) was carried out in order to determine the degree of importance of the individual antagonistic mechanisms (soluble metabolites (SM), volatile antibiotics (VA), laminarinase (L), chitinase (C), and siderophores (S)) in an effort to determine their contribution to the protective effect exhibited by the ***Trichoderma*** spp.. Antagonistic responses observed in both MEA and LNM (Chapter 3) were compared with the % weight loss for the respective basidiomycete treated blocks. The level of lytic enzymes produced in the low nutrient media containing the respective cell wall materials (***N.lepideus*** and ***T.versicolor***) but no glucose (Chapter 3, section 3) was also compared to respective weight loss figures. For the purpose of comparing siderophore production with % weight losses the sizes of halos produced by the different ***Trichoderma*** isolates on CAS and CAS+LNM media (Chapter 3, section 4) were used. The individual % weight loss of the wood blocks obtained with each of the wood decay basidiomycetes in the presence of both live ***Trichoderma*** and after steam sterilisation, was compared individually with each of the antagonistic responses above, detected against each of the basidiomycetes in the two media types (MEA and LNM).

The stepwise regression analysis consisted of a statistical equation or model that considers each different variable (i.e. individual antagonistic response, SM, VA, L, C or S) that influence the value of the % weight loss obtained and incorporates them in an equation in order of their importance in determining the calculated % weight loss. This equation can be represented as follows :

% weight loss = A + V(M1) + W(M2) + X(M3) + Y(M4) + Z(M5)

(A) is the constant for the entire model and V-Z are constants calculated by the statistical analysis (in this particular equation V > W > X > Y > Z) for the individual modes of antagonism M1 to M5, which represent any of the five modes of antagonism mentioned above. The order of occurrence of the antagonistic responses in the above equation is dependent on the basidiomycete causing the degradation and the ***Trichoderma*** species considered.

The importance of each antagonistic mechanism is dependent on the correlation with the % weight loss and is determined by the R^2 value represented as a precentage. The closer the % R^2 value to 100% after consideration of all the mechanisms in the equation the better the model. The greater the increase in the % R^2 value on inclusion of each additional antagonistic response to the model indicates the importance of that factor in determining the % weight loss produced and thereby the extent of any bioprotective effect contributed by that antagonistic mechanism.

The following comparisons of respective weight loss and antagonistic responses were carried out for each basidiomycete - (1) % weight loss in the presence of live antagonist was compared against the antagonistic responses from all ***Trichoderma*** isolates (SM, VA, L, C) in the malt extract medium and the low nutrient medium. (2) % weight loss obtained after killing of the antagonist was compared against antagonistic responses from all ***Trichoderma*** isolates in malt extract medium and low nutrient medium (only SM and VA). As there is no live antagonist present, production of lytic enzymes was not included in these comparisons against weight loss. The influence of siderophores was also compared for the low nutrient medium. Similar comparisons were also made within the species groups, ***T.viride*** and ***T.pseudokoningii*** to determine any particular trend in the mechanisms these groups may apply in protecting wood against these decay fungi.

5.3 Results

5.3.1 % Weight losses of wood blocks in presence and absence of *Trichoderma*

Table 5.3.1 indicates the % weight losses of wood blocks obtained with each of the wood decay fungi after pretreatment with ***Trichoderma*** isolates and also after the antagonist had been killed.

It is evident from the results that in the presence of the live antagonist the wood is generally protected (Table 5.3.1). The highest weight loss value observed was 1.04 % after decay by the brown rot fungus ***N.lepideus***. Weight loss values with the white rot fungus ***T.versicolor*** were however greater with the highest value being 13.18 %. It is generally considered that weight losses of less than 3% are indicative of only the non-structural carbohydrates being removed by the colonising organisms. The results therefore indicate that only B1 (***T.aureoviride***) and B16 (unidentified ***Trichoderma***) have failed to totally protect the blocks against the white rot fungus. It should be noted that the moisture content of all blocks in the experiment fell within a range suitable for decay to take place (data not shown).

The weight loss values are all quite variable within and between species and strains, and on the basis of only the weight loss results no definite conclusion can be drawn about which ***Trichoderma*** spp. has provided the greater protective effect. It is interesting however to note that B1 (***T.aureoviride***) which was selected as a control because of its inability to kill the basidiomycetes in both types of agar media during interaction studies (Chapter 2) shows a slightly higher weight loss than the others against the white rot fungus, however it does give protection against the brown rot fungus.

Weight losses increased considerably in those blocks where the ***Trichoderma*** isolates were killed prior to exposure to the basidiomycete. However these weight losses remained lower than the weight loss of control blocks that showed an average weight loss of 72.6 % for the brown rot and 62.4 % for the white rot fungi. It is evident from the results that almost all the isolates tested produced some reduction in decay susceptibility even when the ***Trichoderma*** was killed.

Trichoderma Isolates	Basidiomycete type			
	N.lepideus		T.versicolor	
	(1)	(2)	(1)	(2)
B1	0.55 (0.27)	54.54 (4.08)	13.18 (2.4)	31.48 (4.1)
B5	0.39 (0.31)	46.74 (3.83)	0.62 (0.35)	32.85 (9.7)
R2	0.87 (0.48)	58.78 (10.1)	0.45 (0.50)	47.88 (9.9)
R7	0.59 (0.48)	56.55 (7.68)	0.28 (0.43)	57.04 (3.71)
R10	0.59 (0.39)	40.87 (9.7)	0.66 (0.44)	44.19 (4.62)
B11	0.70 (0.52)	56.12 (11.89)	2.55 (1.69)	44.01 (11.29)
B13	0.81 (0.46)	49.85 (7.89)	0.06 (0.12)	57.14 (1.01)
B14	1.04 (0.87)	56.50 (4.71)	1.61 (1.78)	55.60 (3.37)
B15	0.63 (0.36)	35.38 (11.9)	0.65 (0.60)	56.84 (1.18)
B16	0.57 (0.36)	59.49 (8.39)	4.49 (3.41)	57.77 (6.59)
Controls	72.66 (2.54)		62.44 (4.25)	

Table 5.3.1 - The % weight losses obtained in blocks subsequently treated with either ***N.lepideus*** and ***T.versicolor*** after (1) pretreatment with live ***Trichoderma*** and (2) pretreatment with ***Trichoderma*** followed by sterilisation. ***Trichoderma*** isolates; B1 - ***T.aureoviride***, B5, R2, R7 and R10 - ***T.viride***, B11 - ***T.harzianum***, B13, B14 and B15 - ***T.pseudokoningii*** and B16 - Unidentified ***Trichoderma***. Standard deviations are in parentheses.

5.3.2 Stepwise Regression Analysis for correlation studies

Tables 5.3.2 (a) and (b) show the % R^2 values obtained during comparison of the % weight losses of wood blocks (with live and killed antagonist) after exposure to each of the basidiomycetes against individual antagonistic responses in malt extract and low nutrient media. Values of % R^2 shown indicate the significance of each individual mechanism or combination of mechanisms to the decay process. The greater the increase in the % R^2 value on addition of each antagonistic response to the model indicates the importance of that mechanism in determining the % weight loss or extent of the protective effect shown by the individual ***Trichoderma*** isolates. The closer the % R2 value to 100% the better the correlation of those mechanisms with the % weight loss in the model.

For both ***N.lepideus*** and ***T.versicolor*** the order of importance of each antagonistic mechanism that influences the protective effect of the ***Trichoderma*** changes in each of the two media i.e. MEA and LNM (table 5.3.2 (a)). The inclusion of siderophore production into the model for the LNM results in a change in the pattern of importance of the other characteristics with ***N.lepideus*** but not with ***T.versicolor***. With ***N.lepideus*** siderophores are identified as the major influence on the weight losses produced (table 5.3.2 (a)). All the final % R^2 values obtained irrespective of presence of live antagonist, increased from MEA to LNM and again to LNM with the inclusion of the siderophore factor, with the exception of weight loss by ***N.lepideus*** with live antagonist.

In comparisons made in the presence of live antagonist (table 5.3.2. (a)) the % R^2 value with ***N.lepideus*** reaches 30% in MEA then falls to 8.07% in LNM and then increases again to 15.65% in LNM with the inclusion of siderophore factor. These values are comparatively lower than that obtained with ***T.versicolor*** where comparisons on MEA gives an % R^2 of 7.05% which increases to 44.71% and 68.55% in LNM and LNM with siderophore factor respectively.

Nl (MEA)		Nl (LNM)		NL (LNM)*	
L	14.95%	VA	0.94%	S	13.99%
L+C	21.88%	VA+C	2.23%	S+SM	14.23%
L+C+VA	28.45%	VA+C+SM	4.38%	S+SM+VA	14.81%
L+C+VA+SM	30.06%	VA+C+SM+L	8.07%	S+SM+VA+C	15.57%
				S+SM+VA+C+L	15.65%
Tv (MEA)		**Tv (LNM)**		**Tv (LNM)***	
SM	4.58%	VA	36.32%	VA	36.32%
SM+VA	6.13%	VA+SM	44.52%	VA+SM	44.52%
SM+VA+C	6.69%	VA+SM+L	44.70%	VA+SM+S	68.47%
SM+VA+C+L	7.05%	VA+SM+L+C	44.71%	VA+SM+S+C	68.55%
				VA+SM+S+C+L	68.55%

Table 5.3.2 (a) - Stepwise regression analysis between antagonistic mechanisms and % wt loss in the presence of live antagonist. % R^2 values shown indicates the significance of each individual mechanism or combination of mechanisms to the decay process.

Key - Nl - ***N.lepideus***, Tv - ***T.versicolor***; MEA, LNM - comparison of weight loss with antagonistic responses as recorded in the malt extract and low nutrient media respectively; (*) Comparison includes results from siderophore production. Antagonistic mechanisms - Soluble metabolites (SM), Volatile antibiotics (VA), Laminarinase (L), Chitinase (C) and Siderophores (S).

Nl (MEA)		Nl (LNM)		NL (LNM)*	
VA	2.19%	VA	3.77%	VA	3.77%
VA+SM	2.26%	VA+SM	5.16%	VA+S	5.72%
				VA+S+SM	7.78%
Tv (MEA)		**Tv (LNM)**		**Tv (LNM)***	
VA	43.31%	VA	46.11%	S	53.55%
VA+SM	45.05%	VA+SM	63.29%	S+VA	93.18%
				S+VA+SM	93.93%

Table 5.3.2 (b) - Stepwise regression analysis between antagonistic mechanisms and % wt loss after killing the antagonists. % R^2 values shown indicates the significance of each individual mechanism or combination of mechanisms to the decay process.

Key - Nl - ***N.lepideus***, Tv - ***T.versicolor***; MEA, LNM - comparison of weight loss with antagonistic responses as recorded in the malt extract and low nutrient media respectively; (*) Comparison includes results from siderophore production. Antagonistic mechanisms - Soluble metabolites (SM), Volatile antibiotics (VA), Laminarinase (L), Chitinase (C) and Siderophores (S).

The failure of the model to give high % R^2 values for decay by ***N.lepideus*** in the presence of live ***Trichoderma*** is accounted by the fact that all the ***Trichoderma*** isolates gave total protection against this fungus. Since no differentiation was observed between weight losses obtained after pretreatment with these antagonists comparison of these results to other mechanisms has limited value.

In order to establish the reasons for the high correlation seen with ***N.lepideus*** in MEA and the lower % R^2 values obtained in LNM and LNM with siderophore factor, the following comparisons were made with the following selected individual and combinations of species groups: 1) ***T.viride*** (4 isolates), 2) ***T.pseudokoningii*** (3 isolates), 3) all isolates excluding B16, the unidentified ***Trichoderma*** isolate.

The results of stepwise regression analysis obtained for the above combinations are shown in Tables 5.3.2 c and d. Analysis of the results from these combinations indicate that the high correlation seen in MEA and low % R^2 in LNM is apparently due to the variability within other different species groups. The results clearly show that in different species groups different mechanisms of antagonism are important in the control of ***N.lepideus***.

Soluble metabolites and the lytic enzymes laminarinase and chitinase play an important role in the antagonism of ***T.viride*** species against ***N.lepideus*** as indicated by their combined % R^2 values (table 5.3.2 c). Since % R^2 value has reached 100% with just three antagonistic responses, these responses are more important than volatiles and siderophores which were not included in the model even though they were considered in the analysis. The results however indicate the following importance in order of mechanisms for ***T.pseudokoningii*** against ***N.lepideus*** in the low nutrient media (table 5.3.2. d), siderophores > enzymes > soluble metabolites > volatiles and a final % R^2 value of 80.6%. The last mechanism, i.e. volatiles however did not contribute very much to the correlation seen with weight loss, as indicated by the very low increase in % R^2 value on subsequent addition of each of these mechanisms. Comparisons that involved the exclusion of B16 from other species groups (data not shown) resulted in similar patterns to those when all isolates were included (table 5.3.2 a).

T.VIRIDE			
NL (LNM)		**NL (LNM)***	
SM	11.63%	SM	11.63%
SM+L	80.54%	SM+L	80.54%
SM+L+C	100%	SM+L+C	100%
TV (LNM)		**TV (LNM)***	
L	54.4%	S	73.3%
L+SM	99.9%	S+C	93.6%
L+SM+VA	100%	S+C+SM	100%

Table 5.3.2 (c) - Stepwise regression analysis between antagonistic mechanisms and % wt loss in the presence of live ***T.viride*** spp. % R^2 values shown indicates the significance of each individual mechanism or combination of mechanisms to the decay process.

Key - Nl - ***N.lepideus***, Tv - ***T.versicolor***; LNM - comparison of weight loss with antagonistic responses as recorded in the low nutrient media; (*) Comparison includes results from siderophore production. Antagonistic mechanisms - Soluble metabolites (SM), Volatile antibiotics (VA), Laminarinase (L), Chitinase (C) and Siderophores (S).

T.PSEUDOKONINGII			
NL (LNM)		**NL (LNM)***	
SM	4.9%	S	20.94%
SM+VA	18.5%	S+L	26.17%
SM+VA+C	35.5%	S+L+C	57.27%
SM+VA+C+L	36.4%	S+L+C+SM	80.4%
		S+L+C+SM+VA	80.6%
TV (LNM)		**TV (LNM)**	
VA	8.66%	VA	8.66%
VA+SM	32.09%	VA+SM	32.09%
VA+SM+C	52.24%	VA+SM+S	52.24%
VA+SM+C+L	53.42%	VA+SM+S+C	96.94%
		VA+SM+S+C+L	97.44%

Table 5.3.2 (d) - Stepwise regression analysis between antagonistic mechanisms and % wt loss in the presence of live ***T.pseudokoningii*** spp. % R^2 values shown indicates the significance of each individual mechanism or combination of mechanisms to the decay process.

Key - Nl - ***N.lepideus***, Tv - ***T.versicolor***; LNM - comparison of weight loss with antagonistic responses as recorded in the low nutrient media; (*) Comparison includes results from siderophore production. Antagonistic mechanisms - Soluble metabolites (SM), Volatile antibiotics (VA), Laminarinase (L), Chitinase (C) and Siderophores (S).

With ***T.versicolor*** the order of importance of the individual mechanisms changes only slightly (table 5.3.2 a) on addition of the siderophore factor which is less important than volatiles and soluble metabolites. The final % R^2 value increases from 44.71% to 68.55%, by the inclusion of siderophores. As the % R^2 value increases only from 68.47% to 68.55%, on addition of the lytic enzyme factor in the model, this implies that these have little influence in the control of ***T.versicolor***. It appears that for ***T.versicolor*** volatiles, soluble metabolites and then siderophores form the order of importance of antagonistic mechanisms. Statistical comparisons within and between species groups of ***Trichoderma*** (table 5.3.2 c and d) indicate very little change in the order of importance to antagonistic mechanisms against the white rot fungi. Volatiles and siderophores seem to be the most common mechanism for almost all the species groups tested, although after these two mechanism the order of importance varies between soluble metabolites and enzymes. ***T.viride*** species however show siderophores to be most important followed by chitinase and then soluble metabolites. The % R^2 value was 73.3% with siderophores which increased to 93.6% with inclusion of chitinase and reached 100% after soluble metabolites were included in the model. This indicates that these mechanism on their own fit the model well and that volatiles and laminarinase enzyme involvement is low.

Comparison of the % weight loss of wood blocks in the absence of the antagonist with the selected antagonistic responses (MEA and LNM) has highlighted the importance of those mechanisms that are likely to be effective even in the absence of the live antagonist (table 5.3.2 (b)). With all the combination of comparisons made between weight loss and antagonistic responses in the MEA and LNM, volatiles are the predominant factor followed by soluble metabolites. On the inclusion of the siderophore factor however the % R^2 value for ***T.versicolor*** reaches a high value of 93 % with, siderophores being the most important single factor. The comparatively higher % R^2 values obtained in the LNM with respect to MEA, is of interest since this indicates that the results of antagonistic responses in LNM correlates better with the % weight loss in wood than that in MEA. Also the % R^2 values with ***T.versicolor*** are considerably higher than that with ***N.lepideus***, which implies that with the latter decay fungus the

antagonistic mechanisms in the absence of the live antagonist do not correlate as well with the weight loss. This is supported by the earlier observations in the presence of live antagonist where the principle mechanisms of inhibition against ***N.lepideus*** were the lytic enzymes while soluble metabolites and volatiles do not appear have a major role to play with the majority of the ***Trichoderma*** species tested (table 5.3.2 c and d). The order of importance of mechanisms against ***T.versicolor*** is siderophores > volatiles > soluble metabolites. Soluble metabolites however do not contribute very much to the model in inhibiting the decay fungus when the antagonist has been killed.

5.4 Discussion

Trichoderma are one of the most favoured biocontrol agents due to their non-demanding nutritional characteristics that enable them to grow on a varied number of substrates; and most important of all, the numerous antagonistic mechanism that they exhibit for their own growth and survival in nature.

Many researchers have been actively involved in elucidating the antagonistic mechanisms of ***Trichoderma*** against plant pathogens. However most of the work that has been published has reported on the use of artificial media and as such the experimentation does not always represent the nutritional consistency of the actual substrate in which the biocontrol agent is to be employed. Therefore the exaggerated effects of antagonistic mechanisms against pathogens that is seen in the artificial media is not reproduced in the field. The nutritional conditions and the actual presence of the pathogen in the vicinity of the antagonist will determine the target specificity and success of the antagonist. Similarly, although much work exists to show the bioprotective effect of ***Trichoderma*** spp. against wood decay fungi in the field and in ***in vitro*** agar plate studies, very little work has been carried out to establish the specific mechanisms that are involved in the inhibition of wood decay fungi. In this study statistical comparison of weight losses as in wood blocks, obtained after exposure to wood decay basidiomycetes, has been compared, with individual antagonistic responses noted in low nutrient media in order to facilitate understanding of the mechanisms that are likely to be of more importance in wood.

Almost all the ten ***Trichoderma*** isolates tested gave a protective effect against both basidiomycetes although there was less variability in the weight losses obtained with ***N.lepideus*** than with ***T.versicolor***. This complete protection against decay fungi was obtained with the wood block test method that is usually adopted for testing of chemical preservatives. Many researchers as already discussed in the introduction, have reported the variable protective effect of ***Trichoderma*** against different target pathogens in ***in vitro*** wood block tests (Morris, 1983; Highley and Ricard, 1988; Morrell and Sexton, 1988). More recently, Bruce ***et al.*** (1991) have shown that wood blocks removed from distribution poles previously treated (after 7 years) with a biological control product (Binab FYT pellets) when exposed in soil block tests to selected basidiomycetes, resisted decay by these organisms.

Killing of the ***Trichoderma*** by steam sterilisation from the pretreated blocks before exposure to decay fungi, resulted in higher weight losses, but were still however 20-30 % lower than the control blocks not treated with any antagonist. This reduction in predicted weight loss could be due to the non-availability of readily usable nutrients that have been exhausted by the ***Trichoderma***, thereby resulting in delayed growth and decay by the basidiomycetes. Bruce and King (1983) however reported that the protective effect of ***Trichoderma*** against the brown rot fungus ***N.lepideus*** was maintained in wood blocks even after ethylene oxide sterilisation and subsequent leaching, implying the presence of a residual component such as soluble metabolites or volatiles that may be the cause of such protective effect. However, the results obtained in this study indicate that the residual protection of wood by ***Trichoderma*** isolates is lost on steam sterilisation, as also observed by Bruce ***et al.***, 1991. This loss of protection against ***N.lepideus*** may be due to that fact that this decay fungus is shown to be inhibited by mainly lytic enzymes by most ***Trichoderma*** isolates and soluble metabolites only play a minor role. With the white rot fungus ***T.versicolor*** however volatiles and soluble metabolites are more important and elimination of these by steam sterilisation results in loss of protection.

It is clear from the stepwise regression analysis that the brown rot and white rot fungi were affected by different antagonistic mechanisms in the presence of the live antagonist. The differences that were observed in the order of importance of different mechanisms seen on MEA and LNM, again stresses the importance of using an appropriate media type for such studies. This illustrates clearly the need to undertake antagonistic studies on a medium more representative of the natural substrate in which the antagonist is to be applied. Since change of media type as noted here clearly alters the relative importance of antagonistic mechanisms within individual ***Trichoderma*** spp., it is probable that the antagonistic responses seen in a nutrient rich media will not be reproduced in wood. Comparison of weight loss with the responses noted in the LNM media including the siderophore factor is therefore likely to be the most realistic correlation that was made to wood. It is evident from the results that in the presence of the live antagonist soluble metabolites, siderophores and lytic enzymes in that order are the principle mechanisms against ***N.lepideus*** by the majority of species groups. Consideration however of all the species gives a very poor correlation to the weight losses obtained as indicated by the low % R^2 value. However this lack of correlation is probably due to a very low variability seen in the weight losses obtained between the isolates, i.e, they all show a very high protective effect.

Against ***T.versicolor*** the principle mechanisms seem to be volatiles, siderophores and to a lesser extent soluble metabolites whereas lytic enzymes seem to play only a minor role in determining the weight losses produced. Consideration of the results of the ***T.viride*** species groups (table 5.3.2 c) indicate however that these antagonists have a high reliance on the lytic enzymes. The order of importance attributed to the individual mechanisms however vary dependent on the decay fungus, confirming the target specificity of certain mechanisms noted in earlier chapters examining individual mechanisms. Target specificity against plant pathogens and wood decay fungi have been noted by some researchers. Sivan and Chet (1986) found that ***T.harzianum*** isolates that showed mycoparasitism by production of lytic enzymes against ***Rhizoctonia solani*** did not exhibit any antagonism against ***Fusarium oxysporum*** and no lytic enzyme production was detected. Similarly, Highley and Ricard (1988) found that ***Trichoderma*** species they tested against wood decay basidiomycetes generally prevented decay by

brown rot rather than white rot fungi. The results presented here also indicate that the ***Trichoderma*** spp. tested show different target specificities and are capable of using different antagonistic responses against different target pathogens.

To establish the importance of the antagonistic mechanisms that are active in the absence of the ***Trichoderma***, weight losses obtained after steam sterilisation were compared with the responses on MEA and LNM. It was evident from the results that volatiles and soluble metabolites are shown to be the important factors in determining weight loss in all comparisons with both decay fungi, even though total protection was not achieved in any sample.

It was evident from the % R^2 values obtained from the comparison of weight loss produced by ***T.versicolor*** after killing of ***Trichoderma*** against responses on LNM, that siderophores play a major role in the antagonism. Volatiles and soluble metabolites also contribute significantly to the increase in % R^2 value and this model gave the highest correlation value obtained of any model including all ***Trichoderma*** isolate comparisons (93%). This high correlation on a media closer to wood may suggest the interaction of these three mechanisms may account for the protective effect in wood. It appears therefore that active inhibition of ***N.lepideus*** is greater in the presence of actively growing ***Trichoderma*** while inhibition of the ***T.versicolor*** may be achieved by metabolites of ***Trichoderma*** even after death of the antagonist. This may indicate that antagonism against the white rot fungus could be achieved at a distance from the interaction site, by the siderophores, volatiles and/or soluble metabolites and may account for the findings of Murmanis ***et al.*** (1988 a) who observed that leakage of cytoplasmic material of lysed basidiomycetes could be observed away from the interaction site and implied the inhibitory volatiles to be the causative agent.

In the previous chapter on siderophores the importance of these iron chelators in the enzymic degradation of wood by both brown and white rot fungi has been discussed. The potential of competition between siderophores of ***Trichoderma*** and basidiomycetes for the available iron in wood as a role in antagonism has also been discussed. If siderophores do indeed play such a role in wood against decay fungi then the results

obtained by stepwise regression imply that these iron chelators may also act in the absence of the antagonist and indeed may play a central role especially in the of control ***T.versicolor***.

Siderophores may also play a major role in the antagonism of ***N.lepideus*** by some ***Trichoderma*** spp. Siderophores of ***T.pseudokoningii*** for example were identified as the most important mechanism for these species followed by the lytic enzymes (table 5.3.2 d). In chapter 3 (section 4) the possible importance of iron acquisition by the ***Trichoderma*** species was discussed, and it was found that among all the species groups tested ***T.pseudokoningii*** showed production of more than one type of siderophore (two phenolates and a hydroxamate) which may enhance their ability to compete for iron.

The results presented in this chapter both collectively for all isolates and for individual species groups, has provided valuable information with regard to the relative importance of antagonistic mechanisms exhibited against the two basidiomycetes. It is evident that there is a definite target specificity towards the basidiomycetes that is indicated by the differences observed in expression of the antagonistic responses. However, the fact that only one representative white rot (***T.versicolor***) and brown rot (***N.lepideus***) fungi were tested does not allow generalisations to be made regarding the antagonistic mechanism against all such basidiomycetes. The study presented above however highlights the importance of assessing individual mechanisms against any specific basidiomycete type. Studies such as these carried out against a larger range of decay organisms will provide a clearer understanding of the antagonism of ***Trichoderma*** against specific basidiomycete fungi.

Knowledge gained from such studies will thereby allow selection of ***Trichoderma*** isolates that are better in expressing those antagonistic mechanisms that are important in the combative strategy against a specific target.

Chapter 6

General Discussion

Chapter 6

6.0 General Discussion

Environmental issues regarding use of hazardous wood preservatives are of increasing concern, and research is now being directed towards applying technologies such as biological control to ameliorate the effects of environmental pollution by such chemicals. Among the vast number of potential antagonists tested ***Trichoderma*** spp. have often been the most favoured biocontrol agents due to the numerous characteristics that enhance their role as efficient antagonists. The biochemical features of ***Trichoderma*** spp. are well documented as are their antagonistic properties which make them potential control agents of many plant pathogenic and wood degrading organisms. Although numerous antagonistic mechanisms of these organisms are known, the degree of importance of these individual mechanism against specific pathogenic fungi still requires to be determined. The study undertaken here has not only evaluated the importance of these individual mechanisms against specific basidiomycetes but has also attempted to highlight the ability of individual antagonistic mechanisms to interact and account for the biocontrol of the decay organisms.

Screening of biocontrol agents is often carried out on inappropriate media such as malt extract agar. The study here has shown the importance of using media (low nutrient media) that is closer to the nutritional consistency of the field substrate i.e., wood. The studies in Chapter 3, sections 1 to 4, examined the following antagonistic responses, soluble metabolites, volatiles, lytic enzymes and siderophores exhibited by ***Trichoderma*** spp. It is evident from the results that these antagonistic responses are dependent on media type. The relative contribution of these individual mechanisms in the low nutrient media is likely to provide a closer representation of that observed in wood. It is obvious therefore that studies carried out in nutrient rich media may result in misleading conclusions with regard to antagonistic potential of certain biocontrol agents and their ability to express the necessary antagonistic traits.

Production of soluble metabolites by the different ***Trichoderma*** isolates was found to be dependent on the media type. Most isolates exhibited higher inhibition of the basidiomycetes by soluble metabolites when grown on the malt medium, this was however not reproduced in the low nutrient media (Chapter 3, section 1). Since the latter medium was devised to be a closer representative of wood in terms of its C:N ratio the results observed in such a media is of more importance in such studies. ***T.viride*** spp. and the unknown ***Trichoderma*** isolate seem to show the highest overall inhibition of the basidiomycetes. It was also evident that the ***Trichoderma*** isolates inhibited the brown rot fungus ***N.lepideus*** more efficiently than the white rot fungus ***T.versicolor***. It is interesting to note that with the stepwise regression analysis comparing % weight loss to antagonistic mechanisms (Chapter 5) the results indicated that soluble metabolites were the most dominant mechanism against the brown rot fungus ***N.lepideus***. This seems to correlate well with the target specificity exhibited towards these fungi by this individual mechanism as noted above.

Volatiles produced by ***Trichoderma*** spp. were also found to be influenced by the nutrient concentration of the media used in the testing (Chapter 3, section 2). In the malt medium inhibition of ***N.lepideus*** was found to be more predominant than that of ***T.versicolor***, however the target specificity against the latter white rot fungus was more evident on the low nutrient media. This clearly illustrated the misinterpretation that is likely to be made, with regard to specificity in inhibition by biocontrol agents on experimentation with inappropriate media. Stepwise regression analysis comparing % weight loss to antagonistic mechanisms, also indicated that volatiles are a dominant mechanism against ***T.versicolor***.

Laminarinase and chitinase enzyme production by the ***Trichoderma*** isolates was also found to be influenced by the media type (Chapter 3, section 3). Though the total activity of the enzymes was higher in the malt extract medium than in the low nutrient media, the situation was reversed on consideration of the specific activity. This implies that though the amount of total protein produced in the malt extract medium is high, the amount of lytic enzyme production is comparatively higher in the low nutrient medium that is a closer representation of the nutritional balance in wood. Growth of

Trichoderma in the low nutrient media with different cell wall material of basidiomycetes revealed that these antagonists exhibit a target specificity that is related to amount of lytic enzyme production. In general the amount of lytic enzymes seemed to be selectively produced in higher levels in the presence of cell wall materials of brown rot fungi than that of white rot fungi, though there is undoubtedly target specificity against individual isolates. Results of the stepwise regression analysis showed (Chapter 5) that inhibition of the brown rot fungus by lytic enzymes is more important in the presence of the live antagonist however this does not seem to be a dominant mechanism against the white rot fungus ***T.versicolor.***

The potential of iron-chelating compounds such as siderophores which have been shown to play an important role in the biocontrol of plant pathogens by bacteria such as ***Pseudomonas*** spp. may also be involved in the biocontrol of wood decay fungi by ***Trichoderma*** spp.. Anke ***et al.*** (1991) have reported the production of hydroxamate type siderophores by ***Trichoderma***. It has been shown in this study however that ***Trichoderma*** spp. are capable of producing both hydroxamate and phenolate type siderophores, where the latter siderophore type was originally detected only with bacteria (Neilands, 1981 a) and more recently with wood decay basidiomycetes (Jellison ***et al.***, 1990). It was observed during this study that a certain degree of variability existed in the amount of production of siderophores as indicated by the halos sizes observed in the CAS agar medium. The number of siderophores produced within individual types (hydroxamates and phenolates) also varied, where an isolate of ***T.pseudokoningii*** showed production of two phenolates unlike the ***T.viride*** isolate tested which only produced one phenolate type siderophore. Since the phenolate type siderophores are known to be more efficient iron chelators than the hydroxamate type siderophores produced by most fungi they may be very valuable for iron competition in wood. Competition for iron between antagonists and wood decay fungi may influence not only their growth due to deprivation of iron that act as co-factors to many proteins and enzymes, but may also have a more direct effect on their wood degrading enzymes as discussed previously (Chapter 3, section 4). Statistical correlation with % weight losses (Chapter 5) indicate that siderophores may play an important inhibitory role mainly against the white rot fungus ***T.versicolor*** and that this mechanism may not be dependent

on the viability of the ***Trichoderma*** isolates. It was interesting to note that a ***T.pseudokoningii*** sp. exhibited production of more than one phenolate type siderophore and stepwise regression analysis also indicated siderophores were the most important antagonistic mechanism exhibited against ***T.versicolor*** by these ***Trichoderma*** spp.

Although there was a certain degree of interspecies and interstrain variability in production of individual antagonistic responses within the ***Trichoderma*** isolates, certain mechanisms were commonly associated with particular species subgroups. In all the antagonistic mechanisms tested it was evident that ***T.pseudokoningii*** and ***T.viride*** species always exhibited a greater antagonistic response against both decay fungi in the low nutrient media and these species may therefore be expected to be effective antagonists against the wood decay basidiomycetes in wood.

Antagonism of ***Trichoderma*** spp. against the mycelial form and spores of basidiomycetes was also tested. It was evident from the results that the specificity of the mechanisms exhibited against the wood decay fungi also vary dependent the morphological forms of the organism (Chapter 4). Soluble metabolites showed complete inhibition of both ***N.lepideus*** and ***T.versicolor***, although some antagonists showed greater target specificity against the brown rot fungus. Volatile antibiotics however demonstrated no inhibition of either basidiomycete, although volatile had been previously shown to inhibit the mycelial form of these two basidiomycetes (Chapter 3, section 2). Since in wood, basidiospores may be one of the first morphological states that may be encountered during invasion the potential of ***Trichoderma*** spp. to inhibit the spores in addition to the mycelial form needs to be carefully considered during the development of any biocontrol system for wood protection.

Analysis of results obtained after statistical comparison imply that ***N.lepideus*** and ***T.versicolor*** are inhibited by the various antagonistic mechanisms to varying degrees. It appears that ***N.lepideus*** inhibition required the presence of the live antagonist, as lytic enzymes seem to be the most important mechanism of control while the white rot fungus ***T.versicolor*** can be affected by the presence siderophores, and other metabolites that are not dependent on the continuous viability of the ***Trichoderma*** spp. It is clear

from this study that more than one antagonistic mechanism is functional in the antagonism by ***Trichoderma*** at any one time. This may be further compounded by the fact that ***Trichoderma*** are capable of enclosing two or more different nuclei in each segment of mycelium separated by a septa, (Sivan and Harman, 1991) and can therefore express varied biochemical traits at different areas of mycelial colonisation. Depending on the phenotypic characteristics at the individual sites of interaction and the signals that are detected from the target organisms, varied antagonistic responses will be activated. This study has examined the production of antagonistic traits under autecological conditions and it must be remembered that this is only one aspect of the interactions between organisms, as in nature behavioural responses of the organisms are allied to the greater competitors. Genotypic characteristics of the ***Trichoderma*** can be manipulated by transformation (Sivan ***et al***, 1992) or protoplast fusion (Stasz ***et al.***, 1991; Sivan and Harman, 1991; Stasz and Harman, 1990) to produce more efficient biocontrol agents. However the antagonistic traits of ***Trichoderma*** and the behavioural responses of the organisms need to be fully understood before any such genetic manipulation for improving the isolates can be undertaken.

Although to date some ***Trichoderma*** spp. have been commercially produced to be used as biocontrol agents against plant pathogens, the difficulties that exist in evaluating the potential of these antagonists at a field scale level against wood decay fungi has limited their production at a commercial level. The statistical model that has been used here to understand the antagonistic mechanisms against specific target fungi can be used to select those ***Trichoderma*** antagonists that fit the model better and are therefore capable of exhibiting antagonism against specific target fungi. The knowledge gained from this study on their antagonistic mechanisms could therefore be used in developing more virulent biocontrol agents. The possibilities of using metabolites similar to that produced by these antagonists, on their own as a bioprotectant can also be explored.

With the increasing concern over environmental pollution with the use of wood preservatives that are facing legislative restrictions, it has become more attractive to adopt safer, more environmentally friendly treatment methods such as biological control or bioprotection or integrated chemical and biocontrol treatment. Only by understanding

the combative strategies of these biocontrol agents can the treatment be made more controllable, reproducible and reliable. Commercial application of ***Trichoderma*** spp. for wood decay control will become more of a common practice only with increasing understanding of the biochemical functioning of such antagonists.

Fungi - "If the Lord Almighty had consulted me before embarking upon the creation, I should have recommended something simpler" - Alfonso X of Castile, 13th century.

"Alfonso X must have been considering *Trichoderma* spp. when he made this statement" - U.Srinivasan 20th Century.

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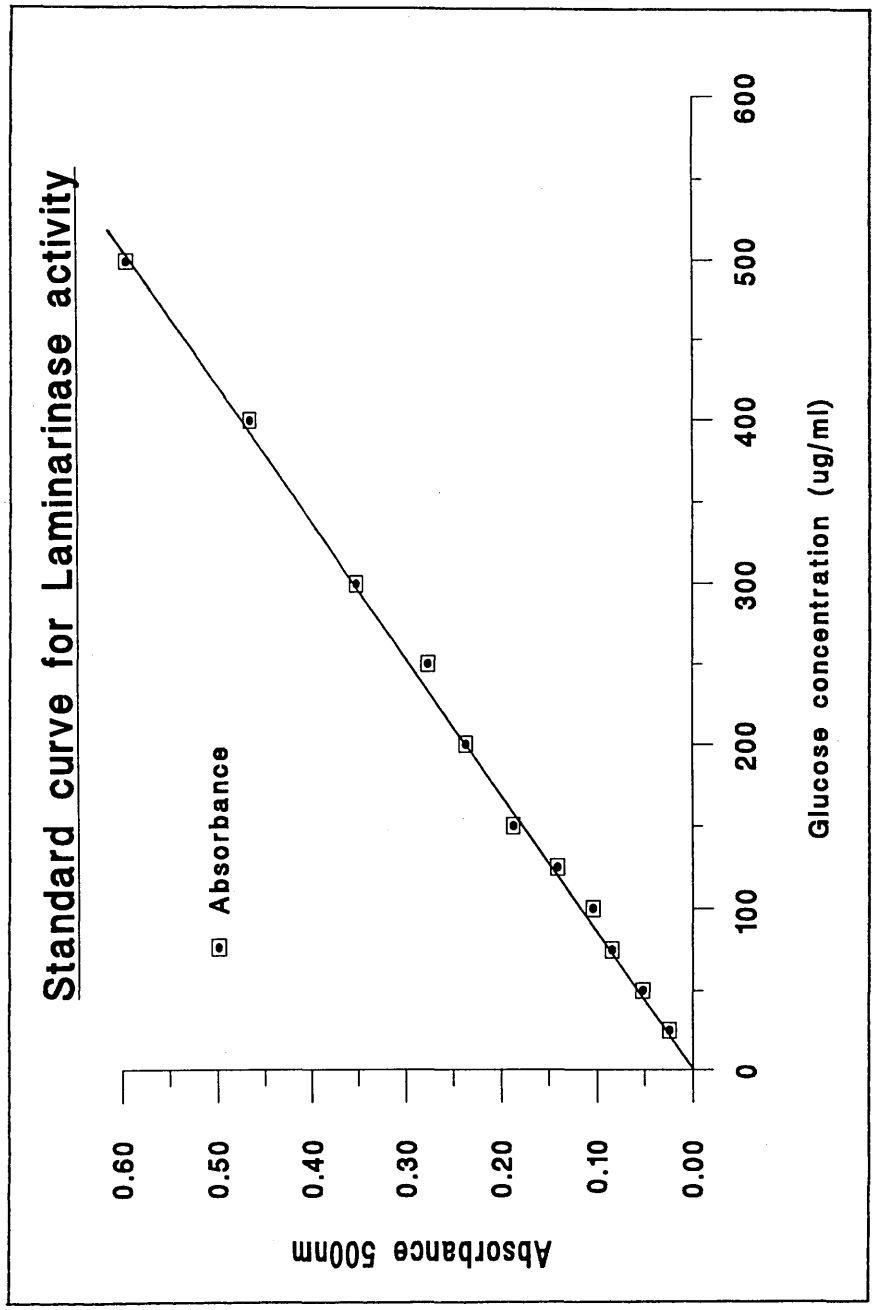
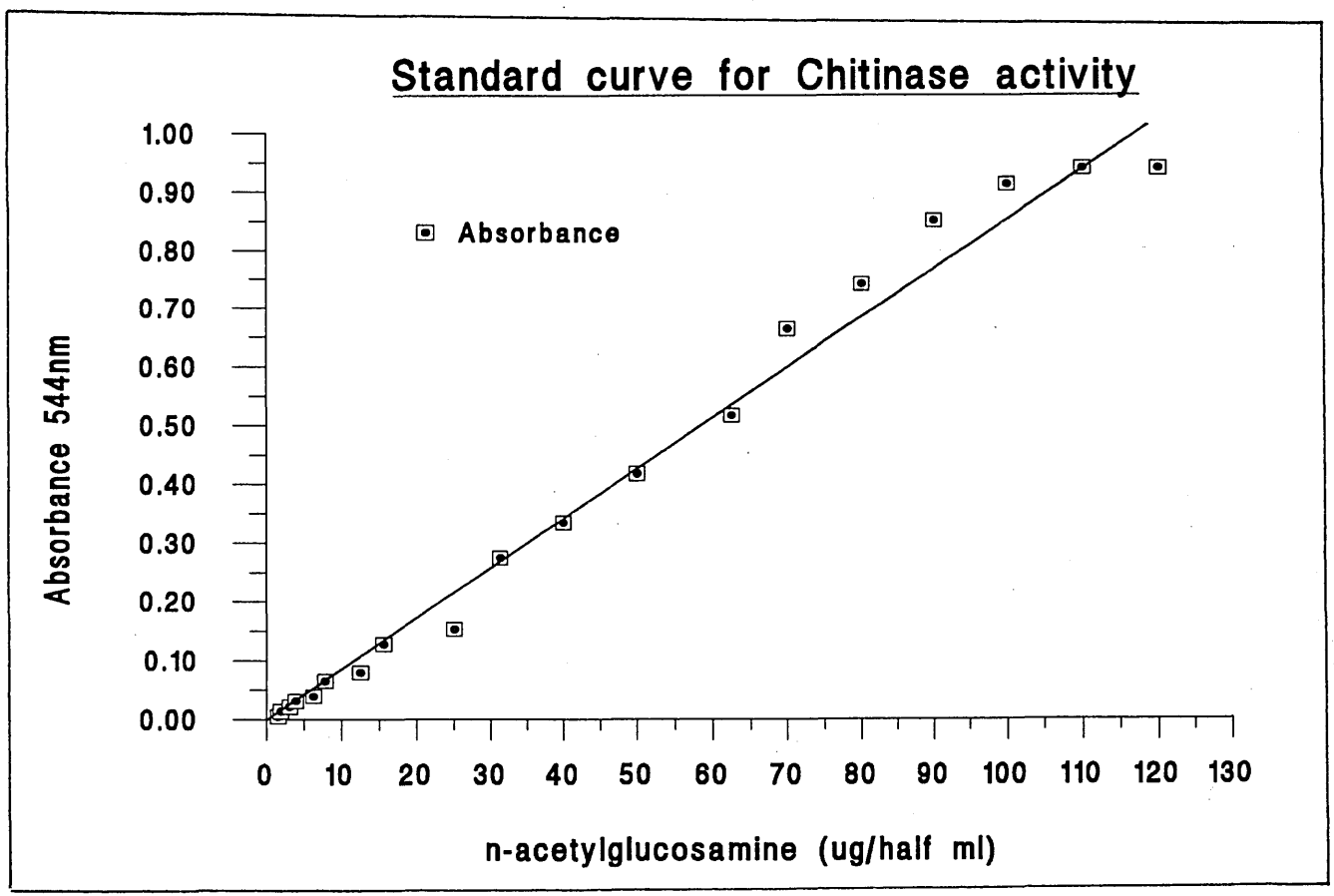
Appendix I

Standard curves for laminarinase and chitinase as assayed by the macroassay is as shown in Figure A and B.

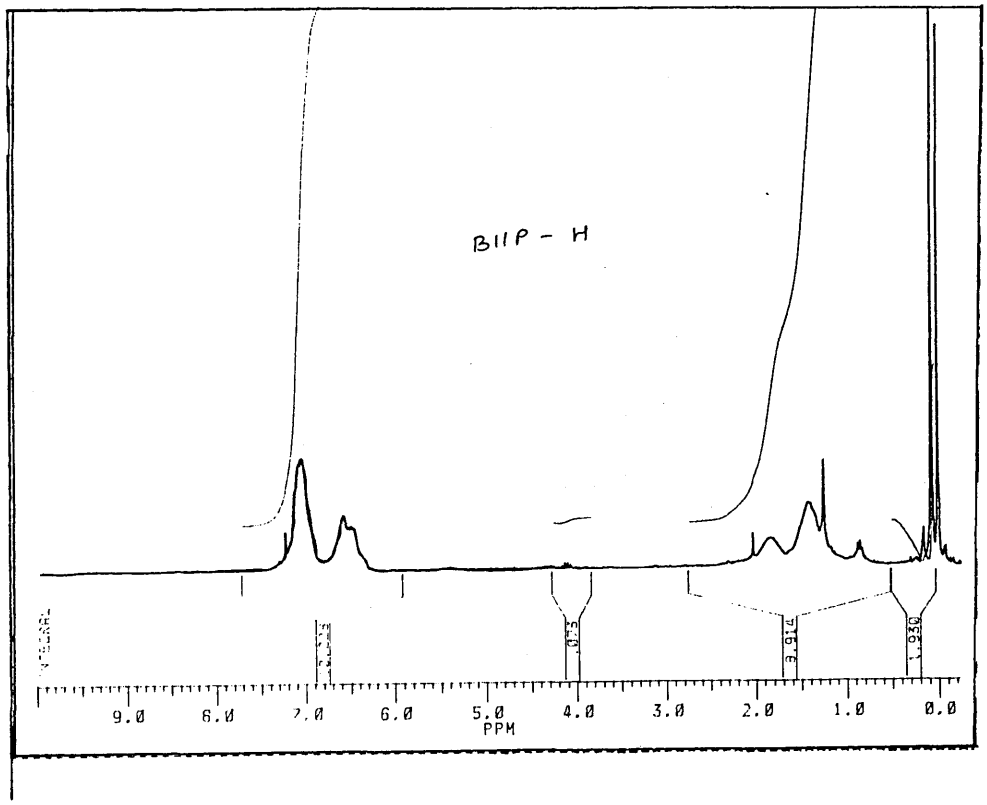


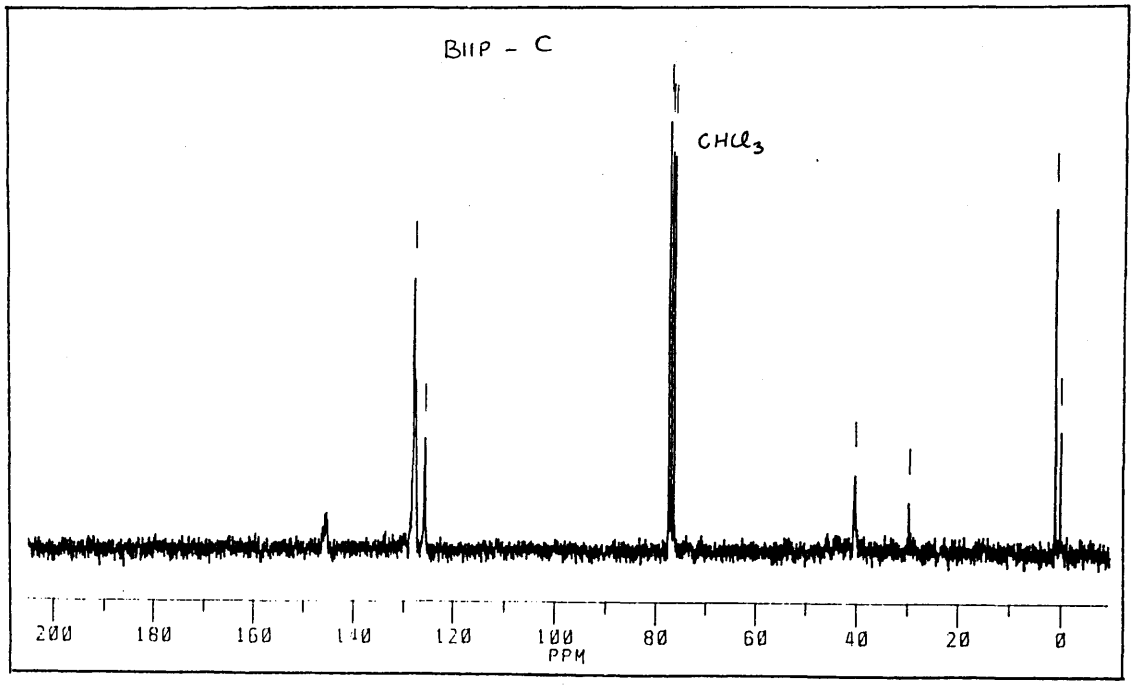

Standard curve for Chitinase activity
Absorbance
Absorbance 544nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
90
100
110
120
130
n-acetylglucosamine (ug/half ml)

Appendix II

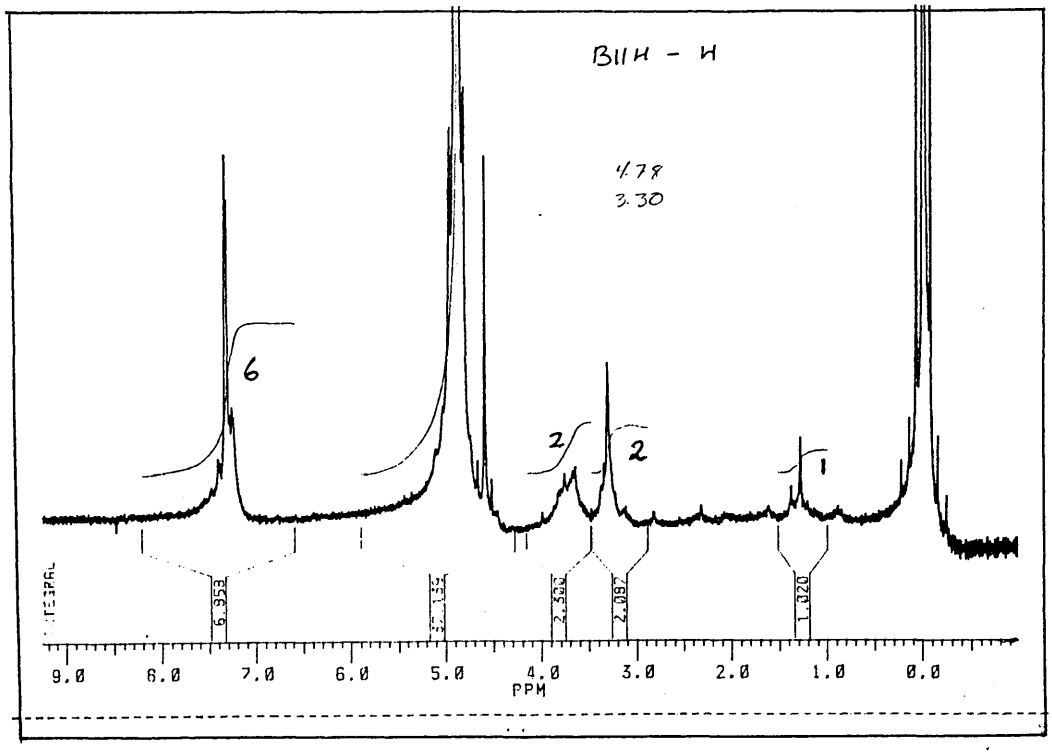
The following spectra are the proton and carbon NMR recording.

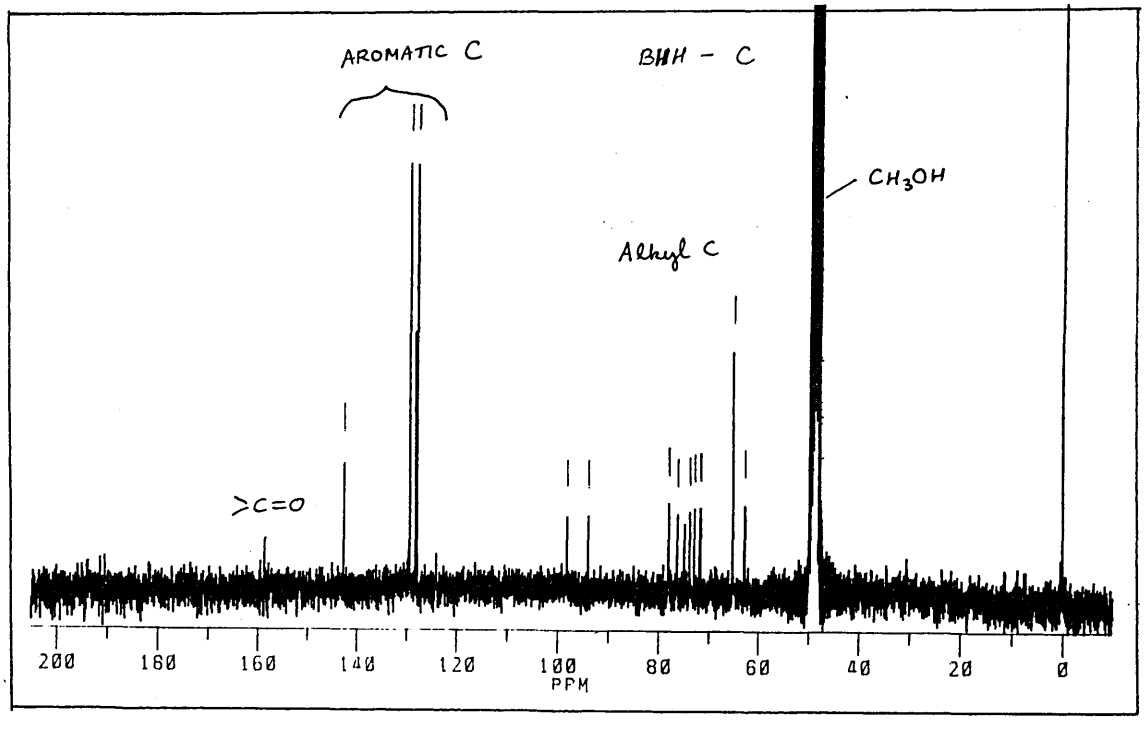
(1) Proton and Carbon NMR of the phenolate siderophores of ***T.harzianum*** (B11).



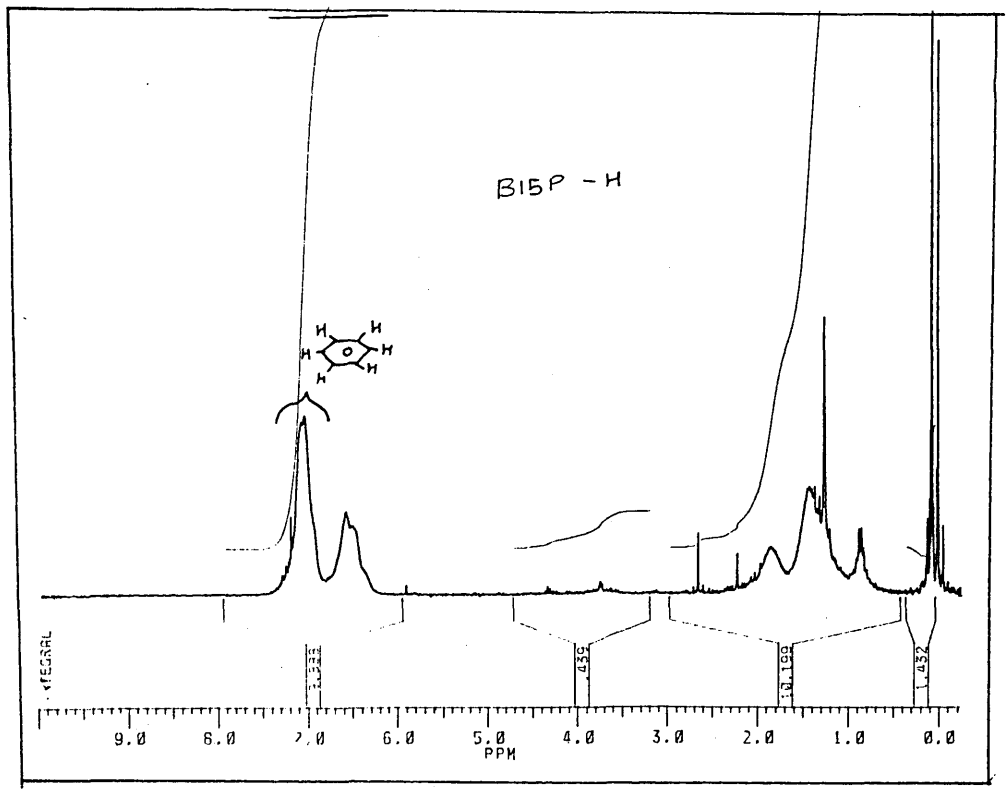


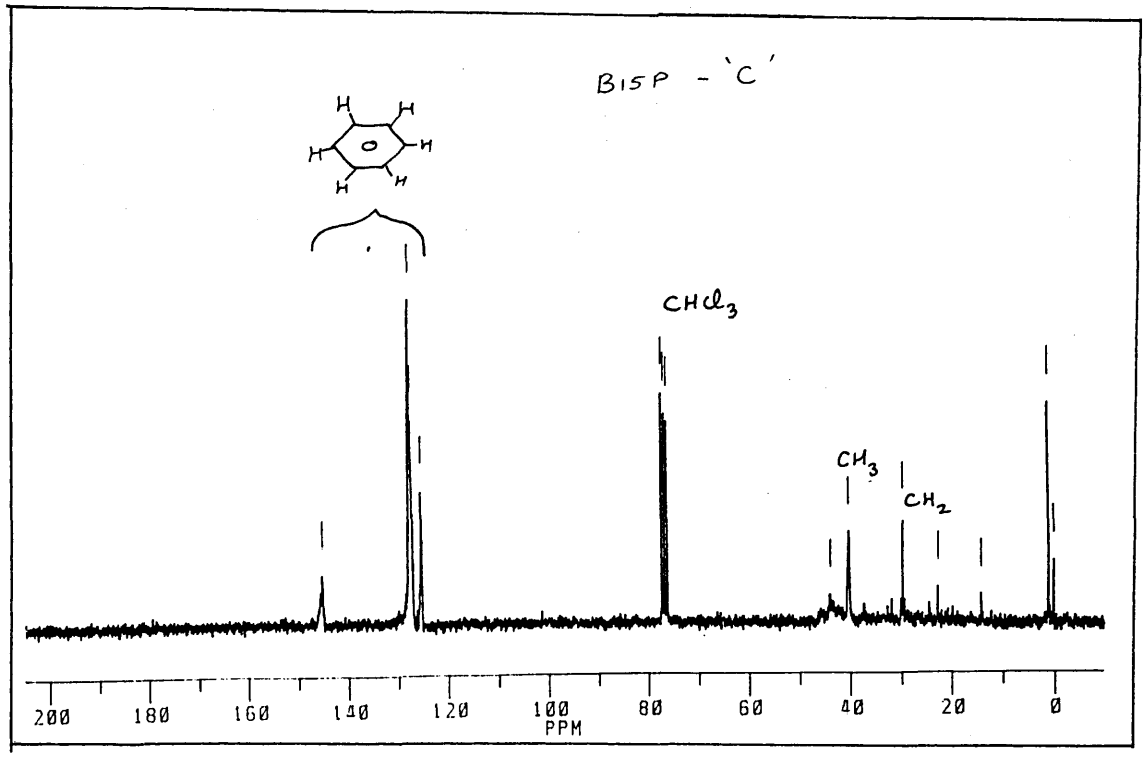
(2) Proton and Carbon NMR of the hydroxamate siderophores of ***T.harzianum*** (B11).

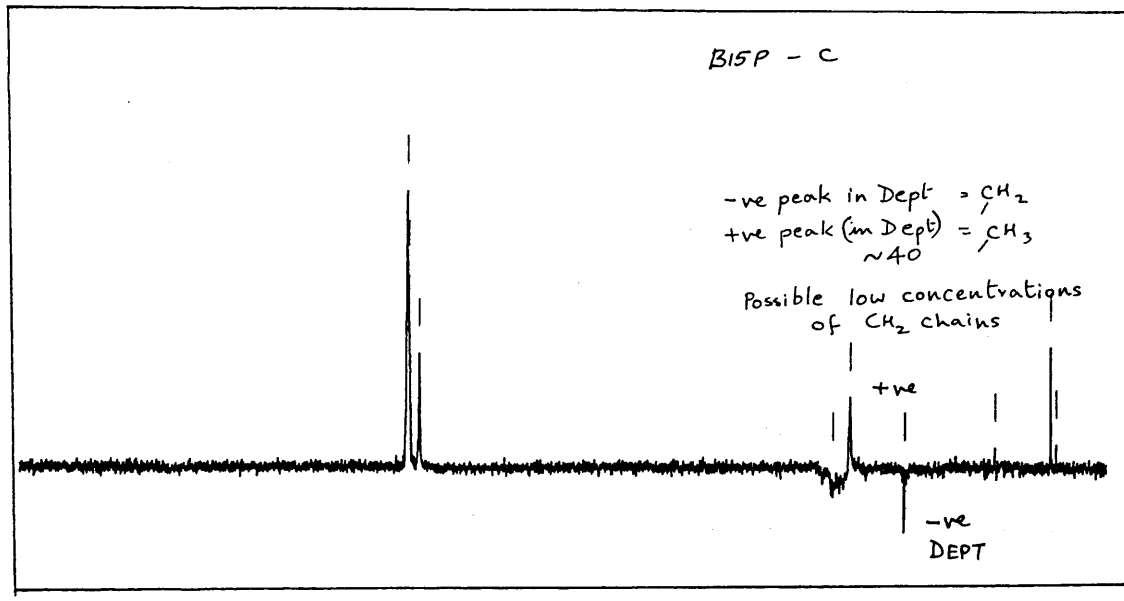




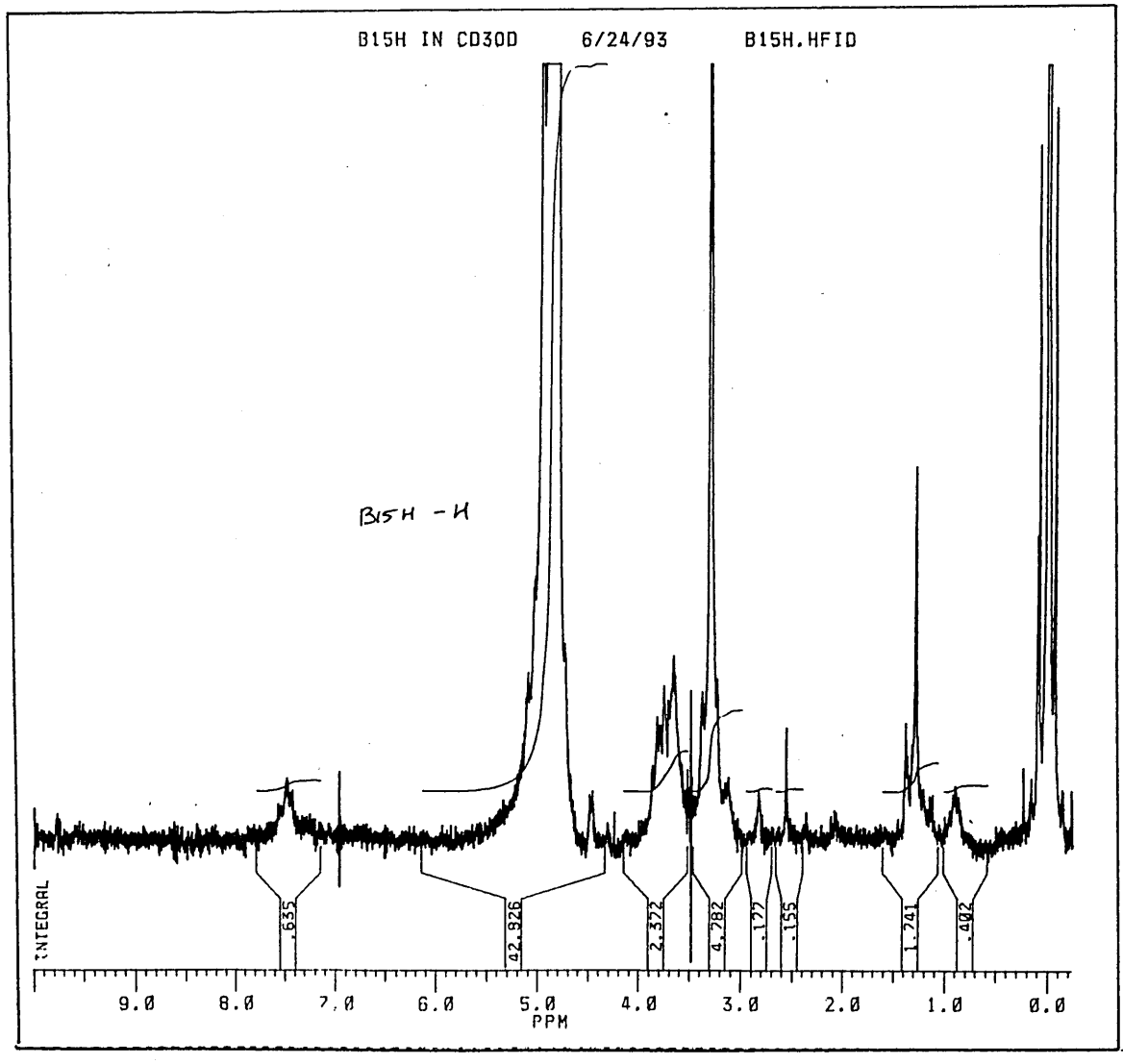
(3) Proton and Carbon NMR and dept of the phenolate siderophores of ***T.pseudokoningii*** (B15).



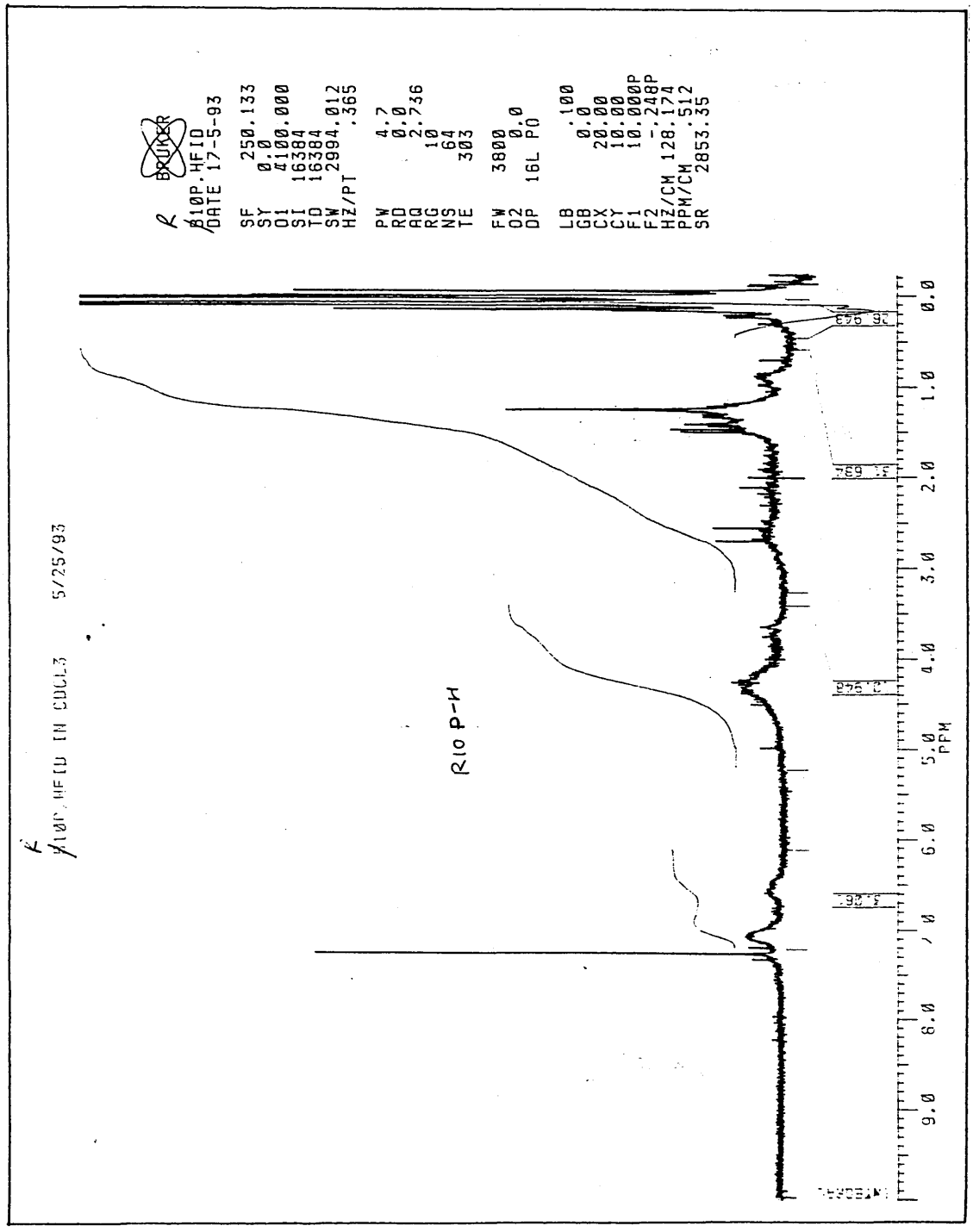




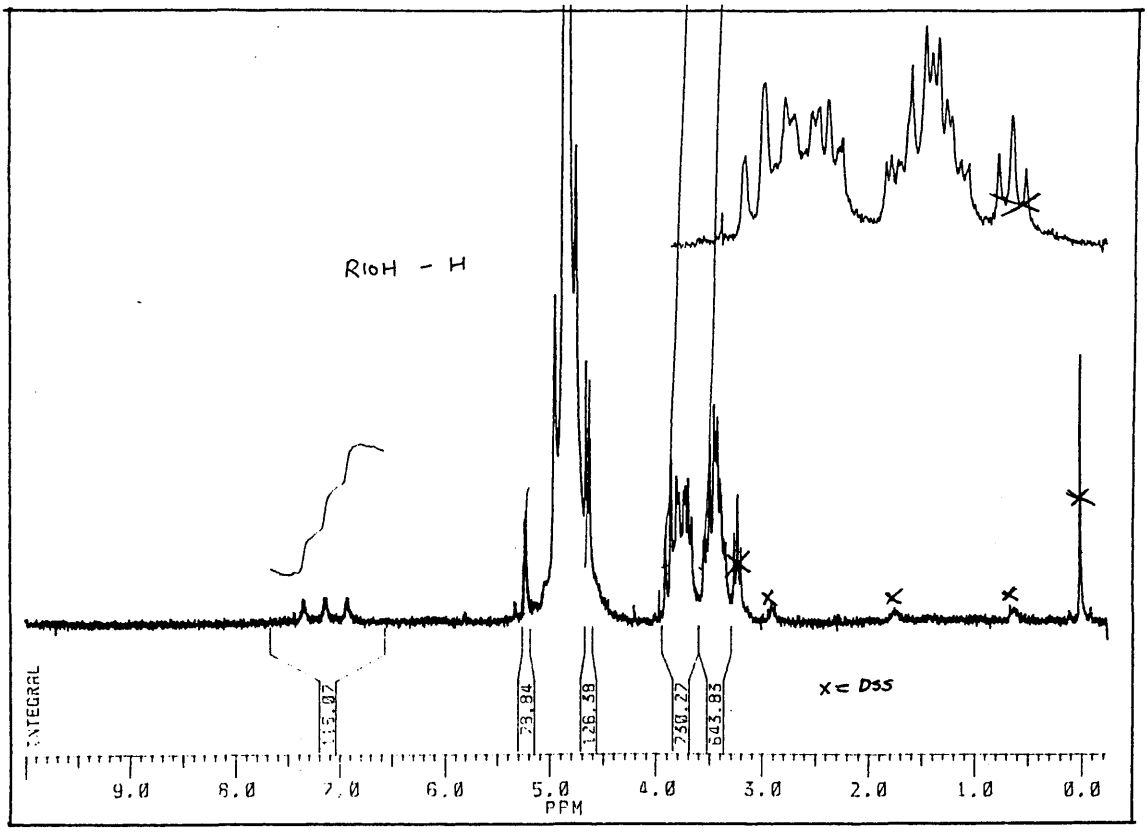
(4) Proton NMR of the hydroxamate siderophores of ***T.pseudokonigii*** (B15).

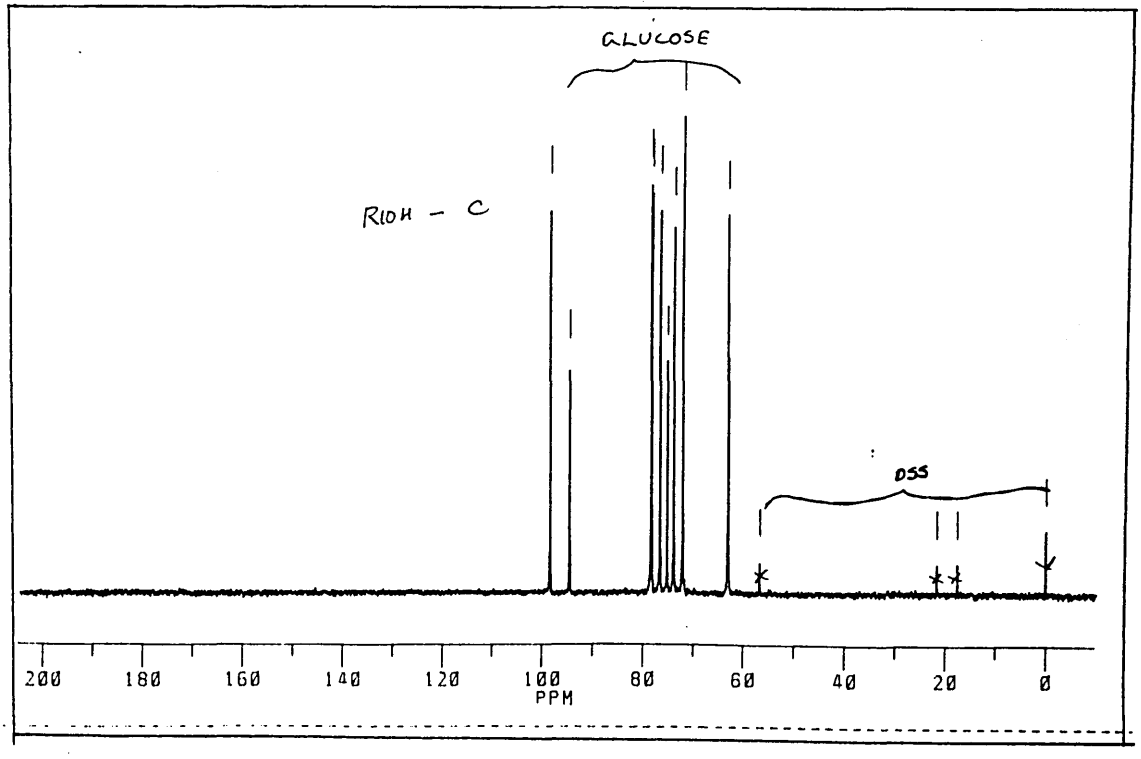


(5) Proton NMR of the phenolate siderophores of ***T.viride*** (R10).



(6) Proton and carbon NMR of the hydroxamate siderophores of ***T.viride*** (R10).





Appendix III

Calculation of WHC of soil

The following procedure for determination of water holding capacity of the soil is based on a method by Bouyoucos (1935). Soil substrate (from, Scottish Crop Research Institute, Invergowrie) with a water-holding capacity between 20 and 40% and pH between 5.0 and 8.0 was used. After breaking up all clumps the soil was mixed and passed through a 2 cm size sieve. This sieved soil was used to fill a small Buchner funnel approximately 5 cm in diameter and 2.5 cm in depth, and fitted with rapid-filtering paper. The soil was made compact by dropping the funnel three times through a height of 1 cm on a wooden tabletop. The soil surface was then levelled by removing excess soil with a spatula at the top of the funnel without further compaction. The filled funnel was then placed in a 400 ml beaker and retained in an upright position by wedges at the sides of the funnel. Water was added to the beaker to a depth slightly beyond the level of the filter paper. The soil was allowed to wet by capillarity to reduce the danger of entrapping air within the column. When the upper soil surface showed signs of wetting, more water was added until the water was level with the upper surface of the funnel. A cover was placed over the beaker, and the soil was allowed to soak overnight (approx 20 hrs). The funnel was then placed in a suction flask which was connected to a vacuum pump, and full suction (133 mBar, 1 Bar = 1 atmospheric) applied for 15 min. During vacuum treatment, the funnel was covered with a moist cloth to prevent evaporation of water from the exposed surface. After 15 min the funnel was removed from the suction flask and the soil scraped out into a receptacle and weighed to obtain the wet weight, W_1 (63.87g) The soil was then oven dried for 24 hr at 105 °C and reweighed, W_2 (50.63g) The soil moisture content at 100% of its water-holding capacity (WHC) was therefore determined based on the ovendry weight of the soil.

$$\% \text{ Moisture content at 100 \% WHC} = \frac{W1 - W2}{W2} \times 100$$

$$= \frac{63.87-50,.63}{50.63} \times 100$$

= 26.15 %.

Preparation of Soil Culture Bottles

140 g of sifted soil was lightly compacted by tapping to half-fill a (250 ml) culture bottle. The water in the soil culture bottle was then made up to 130 % of the water-holding capacity of the soil as follows : The amount of additional water needed was determined by weighing the volume of soil that will be needed to half-fill a culture bottle, i.e., 140 g (W_3). This soil was then dried at 105 ° for 12 hr and reweighed,(W_4 - 120.79 g). The amount of water to be added to each culture was calculated as follows : Water required, g = (100% WHC x 0.013 x W_4) + W_4 - W_3, i.e., (26.15 x 0.013 x 120.79) + 120.79 - 140 = 21.85 ml of water.